

AD_____

Award Number: W81XWH-05-1-0479

TITLE: Development of a Gene Therapy Trial for Metastatic Prostate Cancer

PRINCIPAL INVESTIGATOR: Thomas A. Gardner, M.D.

CONTRACTING ORGANIZATION: Indiana University
Bloomington, IN

REPORT DATE: January 2008

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-01-2008		2. REPORT TYPE Final Addendum		3. DATES COVERED (From - To) 1 JUL 2006 - 31 DEC 2007	
4. TITLE AND SUBTITLE Development of a Gene Therapy Trial for Metastatic Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0479	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Thomas A. Gardner, M.D. E-Mail: thagardn@iupui.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Indiana University Bloomington, IN 46202-5167				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This award was to support the infrastructure and development of a Phase I clinical trial for men with metastatic prostate cancer. The Phase I trial under development employs a prostate restricted replicative adenovirus (PRRA) with excellent preclinical performance in vitro and in vivo in relevant animal models of human prostate cancer. Several components of the statement of work for this award have been completed. The key component of this trial the PRRA, AdIU1, required a slight modification before proceeding with implementation of the trial as submitted. Reconfirmation of the activity of the modified AdIU1 was required prior to moving forward with the clinical trial. Currently with the clinical trial team in place and modified AdIU1 verified and two additional potential prostate cancer therapeutics have been identified further applications to advance this clinical trial program are being prepared.					
15. SUBJECT TERMS Clinical Trial, Metastatic Prostate Cancer, Gene Therapy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 37	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body of report.....	4
Key Award Accomplishments.....	5
References.....	6
Appendix.....	1 and 2

Final Report

Introduction

Men with advanced and metastatic prostate cancer continue to have limited treatment options. Hormonal manipulation, radiation therapy and recently chemotherapy have delayed onset of symptoms and have helped palliated those men with symptoms but novel treatment options are required to advance the care of these men. Gene therapy continues to demonstrate pre-clinical and clinical efficacy in several forms.[1, 2] This award was to facilitate the development of the infrastructure of a Phase I clinical trial for men with metastatic prostate cancer. The Phase I trial under development employs a prostate restricted replicative adenovirus (PRRA) with excellent preclinical performance in vitro and in vivo in relevant animal models of human prostate cancer. [3, 4] Several components of the statement of work for this award have been completed. The key component of this trial the PRRA, AdIU1, required a slight modification before proceeding with implementation of the trial as submitted. The initial form of ADIU1 approached the packaging capacity of the adenoviral vector and decreased the viral yield in larger scale preparation, and may have complicated clinical grade viral production. The modified ADIU1 with a decreased packaging size demonstrate viral yields similar to wild type adenovirus. Reconfirmation of the activity of the modified AdIU1 was required prior to moving forward with the clinical trial. (Appendix 1). The funding was used to support the personnel involved in coordinating the study team to prepare the Phase I application to the DOD to perform manufacturing and product testing and toxicology assessment to initiate a BETA trial with AdIU1, AdIU2 and AdIU3. (Appendix 2) This award has allowed for the clinical trial team to be established while the modified AdIU1 production verified, and additional vectors AdIU2 and AdIU3 completed preclinical testing. Attempts at obtaining funding for the further preclinical and clinical investigations of all three of these agents will be submitted.

Body

The Clinical Trial Development Award has fortified the creation of a Prostate Cancer Clinical Trial Team and has furthered the development of a Vector AdIU1 towards a clinical trial in men with metastatic disease. A Prostate Clinical Trial Team has been developed as a result of this award. The team is comprised of a three Clinician-Scientists Drs. Gardner, Hanh, and Ko representing a genitourinary oncologic surgeon, genitourinary oncologist and genitourinary radiation oncologist, respectively. Collectively, we serve as principle investigators for all the prostate cancer clinical trials open through the Indiana University Cancer Center and are charged by our departments to conduct investigator-initiated and pharmaceutical trials for men with prostate cancer. We have completed several trial with the joint assistance of the Clinical Research Office of the IUCC and the Department of Urology Clinical trials Office including Rhoda Loman and Josie Bergin as Research coordinator and administrator.

The progress on development of AdIU1 clinical trial had been delayed because of an initial production problem associated with the former version of AdIU1. This vector has been modified and is able to be produced at a similar level to wild type production while maintaining the prostate cancer specific oncolytic ability demonstrated by the initial version of the AdIU1. These preclinical investigations have confirmed the viability of AdIU1 as a novel therapeutic for men with advanced prostate cancer. The Award has allowed for maintenance of the clinical trial team and preparation of grants to obtain funding for pre-clinical toxicology and potential BETA (Biological Efficacy and Toxicology Assessment) trial design for AdIU1, AdIU2 and AdIU3. Appendix 2 is the body of

grant that will be submitted in 2009 for Quick Trials Award, DOD Translational Award and Prostate Foundation Award. Obtaining funding to support this further development will allow for submission to NCI or DOD to conduct several clinical trials for men with prostate cancer.

Key Award Accomplishments

- A. Completion of the Study Team with monthly meeting to discuss trial development
 - 1. Urologist: Thomas A. Gardner, M.D.
 - 2. Oncologist: Noah Hanh, M.D.
 - 3. Radiation Oncologist: Song-chu Ko, M.D./Ph.D.
 - 4. Research Coordinator: Rhoda Loman
 - 5. Administrator: Josie Bergin
- B. Confirmation of simplified product development
(Appendix I)
- C. Preparation of Grant Application to obtain additional funding for toxicology testing.
(Appendix II)

References:

1. Freytag, S.O., et al., *Five-year Follow-up of Trial of Replication-competent Adenovirus-mediated Suicide Gene Therapy for Treatment of Prostate Cancer*. Mol Ther, 2007. **15**(3): p. 636-42.
2. Desai, P., et al., *Future innovations in treating advanced prostate cancer*. Urol Clin North Am, 2006. **33**(2): p. 247-72, viii.
3. Lee, S.J., et al., *Novel prostate-specific promoter derived from PSA and PSMA enhancers*. Mol Ther, 2002. **6**(3): p. 415-21.
4. Li, X., et al., *Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer*. Cancer Res, 2005. **65**(5): p. 1941-51.

ORIGINAL ARTICLE

Enhanced combined tumor-specific oncolysis and suicide gene therapy for prostate cancer using M6 promoter

M Ahn^{1,2}, S-J Lee^{1,2}, X Li^{1,2}, JA Jiménez^{1,2,3}, Y-P Zhang^{1,2}, K-H Bae^{1,2}, Y Mohammadi⁴, C Kao^{1,2,3} and TA Gardner^{1,2,3}

¹Department of Urology, Indiana University School of Medicine, Indianapolis, IN, USA; ²Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN, USA; ³Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, USA and ⁴Department of Biology, Indiana University School of Medicine, Indianapolis, IN, USA

Enzyme pro-drug suicide gene therapy has been hindered by inefficient viral delivery and gene transduction. To further explore the potential of this approach, we have developed AdIU1, a prostate-restricted replicative adenovirus (PRRA) armed with the herpes simplex virus thymidine kinase (HSV-TK). In our previous Ad-OC-TK/ACV phase I clinical trial, we demonstrated safety and proof of principle with a tissue-specific promoter-based TK/pro-drug therapy using a replication-defective adenovirus for the treatment of prostate cancer metastases. In this study, we aimed to inhibit the growth of androgen-independent (AI), PSA/PSMA-positive prostate cancer cells by AdIU1. *In vitro* the viability of an AI- PSA/PSMA-expressing prostate cancer cell line, CWR22rv, was significantly inhibited by treatment with AdIU1 plus GCV ($10\mu\text{gml}^{-1}$), compared with AdIU1 treatment alone and also cytotoxicity was observed following treatment with AdIU1 plus GCV only in PSA/PSMA-positive CWR22rv and C4-2 cells, but not in the PSA/PSMA-negative cell line, DU-145. *In vivo* assessment of AdIU1 plus GCV treatment revealed a stronger therapeutic effect against CWR22rv tumors in nude mice than treatment with AdIU1 alone, AdE4PSESE1a alone or in combination with GCV. Our results demonstrate the therapeutic potential of specific-oncolysis and suicide gene therapy for AI-PSA/PSMA-positive prostate cancer gene therapy.

Cancer Gene Therapy (2009) 16, 73–82; doi:10.1038/cgt.2008.59; published online 5 September 2008

Keywords: suicide gene therapy; M6 prostate-specific promoter; HSV-TK; prostate cancer

Introduction

Prostate cancer is still the leading cancer diagnosed in American men. The incidence of prostate cancer is age-dependent and has steadily increased over the last several decades.¹ Localized prostate cancer can be managed effectively with surgery or radiation, whereas advanced and metastatic disease eventually progresses to an androgen-independent (AI) state with limited treatment options. The aging population of men with an increase in prostate cancer incidence combined with an absence of successful therapies for advanced disease require the development of novel therapies.

Suicide gene therapy is an attractive approach to increasing drug selectivity toward cancer cells. Tumor-specific suicide gene therapy using a tissue-specific

promoter is a rational treatment strategy for prostate cancer.^{2–6} Herpes simplex virus thymidine kinase (HSV-TK)-based suicide gene therapy has been used to target prostate cancer for over a decade.^{7–10} The pro-drug, ganciclovir (GCV) is phosphorylated by HSV-TK to its monophosphate form, which is rapidly converted to di- and triphosphate forms by cellular kinases, the latter of which is toxic to cells. The GCV triphosphate is incorporated into DNA during cell division, causing single-strand DNA breaks and inhibition of DNA polymerase^{10–12} and causes DNA chain termination, which leads to programmed cell death.

Tumor-specific oncolytic adenoviruses have been effective and safe treatment options for patients with metastatic disease. Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors. Through the use of prostate-specific promoters and enhancers, the expression of a therapeutic gene or adenoviral replication factor can be limited to cells that contain the appropriate activators and transcription factors. Currently, kallikrein 2, PSA, rat probasin and osteocalcin (OC) are each under extensive

Correspondence: Dr TA Gardner, Associate Professor of Urology, Microbiology and Immunology, Indiana University School of Medicine, 1001 W 10th St., OPW320, Indianapolis, IN 46202, USA. E-mail: thagardn@iupui.edu

Received 10 September 2007; revised 24 February 2008; accepted 16 April 2008; published online 5 September 2008

investigation as regulators of prostate-restricted replication adenoviruses.^{13–18} We have demonstrated that both the prostate-specific antigen (PSA) and OC promoters could transcriptionally regulate the HSV-TK gene in a prostate-specific manner both *in vitro* and *in vivo*.¹⁹ This tissue-specific HSV-TK production combined with pro-drug administration inhibited the growth of androgen-independent (AI)-PSA-producing cells *in vitro*, in animal models of human prostate cancer and in patients with prostate cancer enrolled in a phase I clinical trial of OC promoter-based HSV-TK gene therapy.^{19,20} Others have demonstrated similar *in vitro* and *in vivo* efficacy as well as safe administration to men with locally advanced and metastatic prostate cancer.^{21,22} More recently, Freytag *et al.*^{23–25} demonstrated the safety and efficacy of a conditionally replicating, non-tissue-specific adenovirus containing the suicide genes *TK* and *CD* when combined with external beam radiation therapy. Although Ad5-CD/TKrep/pro-drugs/radiation therapy demonstrated a promising result in locally recurrent prostate cancer, the expression of the CD/TK fusion gene under the control of strong universal CMV promoter also showed lack of tissue specificity, which severely impairs the safety of this virus. Besides, Ad5-CD/TKrep is based on the E1B 55k deleted virus, del520/ONXY-015. Recent studies indicated that the replication of del520 virus is p53-independent.^{26–29} In addition, del520 exhibits substantial replication in certain normal cells. These complications will potentially limit the use of Ad-CD/TKrep. Nevertheless, Ad5-CD/TKrep warrants further development as a suicide gene therapy vector.

We recently developed a prostate-specific chimeric enhancer, *PSES*, by combining enhancers from *PSA* and *PSMA* genes. PSA and prostate-specific membrane antigen (PSMA) are prostate-specific biomarkers expressed by the majority of prostate tumors and non-cancerous epithelium. The main prostate-specific enhancer activity of the PSA enhancer core is located in a 189 bp region called AREc3, and the main prostate-specific enhancer activity of the PSMA enhancer core is located in a 331 bp region called PSME(del2). The combination of these two regulatory elements, AREc3 and PSME(del2), called prostate-specific enhancing sequence (*PSES*), showed high activity specific to PSA/PSMA-positive prostate cancer cells, regardless of androgen status.³⁰ This *PSES* promoter has been used to control the replication of a PRRA, which demonstrated prostate-specific replication and therapeutic efficacy both *in vitro* and *in vivo*.³¹ We also made a shorter form by deleting the L2 and L5 regions in AREc3 and replacing the 90 bp proximal region of PSME with a simple AP-3 binding site. This version of *PSES* was called M6. For this study, we developed a novel HSV-TK-armed tissue-specific replicative adenovirus, AdIU1, using the *PSES* promoter to drive the expression of adenoviral *E1a* and *E4* and the M6 promoter to drive the expression of adenoviral *E1b* and HSV-TK. AdIU1 demonstrated selective cytotoxicity toward AI PSA/PSMA-expressing prostate cancer both *in vitro* and *in vivo*.

Materials and methods

Cells and cell culture

HER 911E4 cells, derived from adenoviral *E1* (bp 79–5789)-immortalized HER 911 (human embryonic retinoblastoma) cells, express adenoviral E4 proteins under control of the *tetR* promoter. HER 911E4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), hygromycin B (0.1 mg ml⁻¹; Calbiochem, San Diego, CA) and doxycycline (2 µg ml⁻¹; Sigma, St Louis, MO). AI, androgen receptor (AR) and PSA/PSMA-positive prostate cancer cell lines C4-2 and CWR22rv, and AI, AR and PSA-negative cell lines DU-145 and PC3 were cultured in RPMI 1645 supplemented with 10% FBS and 1% P/S. Human renal cell carcinoma RCC29, human testicular cancer cell line Tera-1 and human colon cancer cell line HT-29 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% P/S. The breast cancer cell line MCF 7 was cultured in MEM with 10% FBS and 1% P/S with 1 mM sodium pyruvate. HER 293 cells were cultured in MEM with 10% FBS and 1% P/S and 0.1 mM non-essential amino acids. The cells were maintained at 37 °C in a 5% CO₂ incubator.

Luciferase assay

The tissue-specific activity of M6 was investigated by transient transfection for luciferase assay. A certain number of cells (2 × 10⁵ cells per well) were plated for 24 h. Plasmid DNA, pGL3/M6/TATA and pGL3/TATA were delivered into the cells with DOTAP (Roche, Indianapolis, IN) following the manufacturer's protocol. DNA (0.5–1 µg) was mixed with lipid at room temperature before addition to a well containing 1 ml of serum-free and phenol red-free RPMI 1640 medium. After 15 min, DNA–lipid complexes were added to the well and incubated for 5 h at 5% CO₂ and 37 °C. DNA–lipid-containing medium was then replaced with 1 ml culture medium. After 2 days, the cells were collected and lysed in 250 µl passive lysis buffer (Promega, Madison, WI). Cell lysates were vortexed for a few seconds and spun for 3 min. A supernatant (10 µl) was mixed with 50 µl of luciferase substrate (Promega, Madison, WI) and measured with a femtometer (Zylux, Germany). The luciferase activity was determined by being divided by the basal activity represented by transfection of pGL3/TATA.

Construction of the prostate-restricted replicative adenovirus AdIU1

The construction of the backbone for AdE4PSESE4 was described earlier.³¹ To construct AdIU1, the *CMV-EGFP* expression cassette in AdE4PSESE1a was replaced by an M6-HSV-TK expression cassette. HSV-TK and *E1b* in the left arm was under the transcriptional control of M6 and *E4* and *E1a* in the right arm was controlled by a *PSES* promoter. Figure 1 illustrates the structure of each virus used in this study. The adenoviral genome was released from the cloning vector by digestion with Pac I restriction

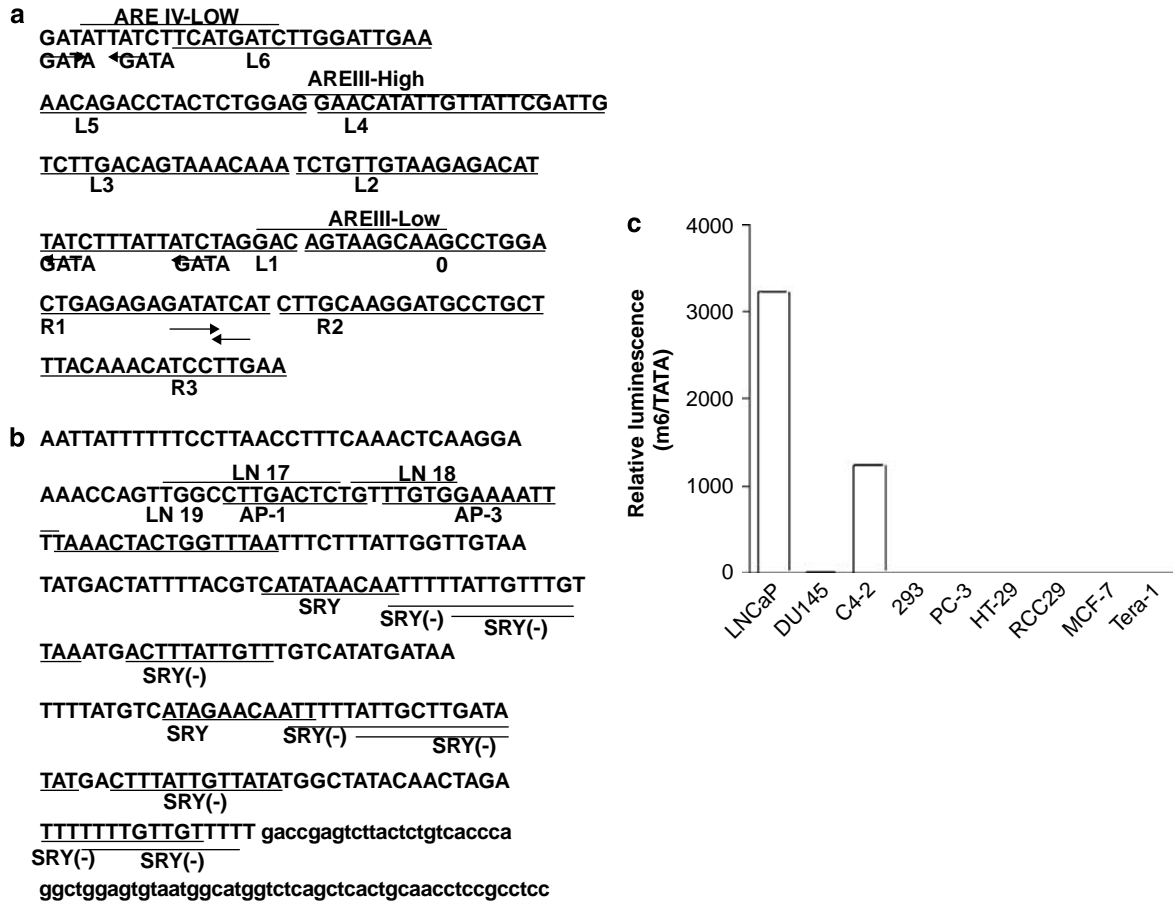


Figure 1 (a) Sequences of the enhancer core of the PSA gene, AREc3, located in the 4.3 kb upstream of PSA promoter. Sequence analysis of AREc3 revealed six putative GATA transcriptional factor-binding sites besides the reported three androgen-response elements. (b) Sequences of PSME located in the third intron in the PSMA gene (*FOLH1*). PSME is characterized by the repeat sequence (marked by underline and bold). Several potential transcription factor-binding sites, such as AP-1, AP-3 and SRY/SOX, are indicated. (c) pGL3/m6/TATA (0.5 µg) was transfected into various cell lines (2×10^5 cells for each). After 2 days, cells were harvested, lysed with passive lysis buffer and analyzed for luciferase activity. This experiment was conducted in the absence of androgen. Luciferase activity was determined by being divided by the basal activity represented by transfection of pGL3/TATA. pGL3/m6/TATA is active only in PSA/PSMA-positive LNCaP and C4-2 cells.

enzyme and transfected into HER 911E4 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The plate was incubated at 37 °C under 5% CO₂ for 7–10 days after transfection until a cytopathic effect was observed. AdIU1 was further amplified in HER 911E4 cells. The recombinant adenoviruses were purified by CsCl gradient centrifugation. All gradient-purified viral stocks were dialyzed in a dialysis buffer (1 mM MgCl₂, 10 mM Tris-HCl (pH 7.5) and 10% glycerol) for 24 h at 4 °C, with three buffer changes. Aliquots of purified virus were stored at –70 °C. The viral titer was determined by Adeno-XTM Rapid Titer system (BD Biosciences, Palo Alto, CA) following the manufacturer's protocol. Briefly, a dilution of the viral stock in question was used to infect HER 911 E4 cells. Forty-eight hours later, these cells were fixed and stained with antibody specific to the adenovirus hexon protein. A signal was detected after a secondary antibody conjugated with horseradish peroxidase amplified the signal of the anti-hexon antibody. Subsequent exposure to a

metal-enhanced diaminobenzidine substrate turned the infected cells dark brown. Then the titer of the stock in question could be determined by counting the number of brown cells in a given area. Each stained cell corresponded to a signal infectious unit (IFU).

Viral replication assay

CWR22rv, C4-2, PC-3 and DU-145 cells were seeded in 6-well plates (1×10^6 cells per well) 1 day before to viral infection and subsequently infected with AdIU1 or AdE4PSESE1a (2 IFU per cell). The media were changed 24 h after infection, and the viral supernatants were harvested 3 days after infection. The cells were examined under light microscopy daily for up to 5 days. Then the titers of the harvested viral supernatants were determined by titer assay. HER 911E4 cells were seeded in 96-well plates (5×10^3 cells per well) 1 day before infection. The cells were infected with serial volume dilutions of the harvested supernatants, ranging from 1 to 10^{-11} µl per well, with each row of 8 wells receiving the same dose of

virus. The media were changed on day 4, and the cells were examined under the microscope on day 7. The dose of the produced viruses was represented as an LD₅₀ value, the dilution factor that caused a cytopathic effect in at least 4 wells of cells in a single row on a 96-well plate by day 7.

Western blot analyses for E1a and HSV-TK expression

To detect the tissue-specific expression of Ad5E1a and HSV-TK, 1×10^6 cells in 60 mm dishes were infected with AdIU1. Each cell line was infected with standardized doses of virus.³¹ Cells were harvested and lysed in 100 μ l of cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 μ g ml⁻¹ Aprotinin, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin, 1 mM Na₃VO₄ and 1 mM NaF) 48 h post-viral infection. Lysates were centrifuged at 14 000 r.p.m. for 20 min and the supernatants were collected. Protein concentration was estimated by dye binding assay (Bio-Rad, Hercules, CA). Protein (25 μ g) was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and the membrane was probed with antibodies reactive to Ad5E1a protein (BD Bioscience) or TK polyclonal serum (provided by M Black, Department of Pharmaceutical Sciences, Washington State University, Pullman, WA). and visualized by an enhanced chemiluminescence kit (Amersham Life Science, Piscataway, NJ).

Dose-dependent in vitro cell-killing assay

CWR22rv and DU145 cells were seeded onto 24-well plates at a density of 1.5×10^5 or 1×10^4 cells per well respectively. After 24 h, the cells were infected with 0.0002–2 IFU per cell of AdIU1 or AdE4PSESE1a. Twenty-four hours after infection, the media were removed and replaced by fresh media with or without GCV (10 μ g ml⁻¹). Media with or without GCV were changed every 2 days. Viable cells were analyzed by crystal violet assay 7 days post-infection.

Time-dependent in vitro killing assay

CWR22rv and DU-145 cells were plated in 24-well plates. Cells were divided into four treatment groups, no treatment, AdIU1 (0.2 IFU per cell), GCV and AdIU1 (0.2 IFU per cell) plus GCV. The media were changed 24 h after infection, and GCV (10 μ g ml⁻¹) was added 24 h after media change. Cell viability was analyzed on days 1, 3, 5 and 7 by crystal violet assay.

In vivo evaluation of AdIU1 therapy

All animal methods and procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC). CWR22rv xenografts were established by injecting 2×10^6 cells subcutaneous (s.c.) in the flanks of 6-week-old male, athymic nude mice. The injected mice were castrated 3 days after cell injection. Mice with similar tumor sizes (3–5 mm) were divided into four groups receiving, AdE4PSESE1a (control PRRA), AdE4PSESE1a plus

GCV, AdIU1, or AdIU1 plus GCV treatment. Virus particles (2×10^9) of either AdE4PSESE1a or AdIU1 in 100 μ l PBS were injected intratumorally. Five days after virus injection, GCV (40 mg kg⁻¹ body weight) was administered intraperitoneally two times daily for 10 days. Tumor sizes were measured every 5 days, and the following formula was applied to calculate tumor volume $\text{length} \times \text{width}^2 \times 0.5236$. Mice were killed and tumors harvested for histological examination 30 days after injection.

Histology and immunohistochemistry

Tumors were harvested, immediately fixed in formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin according to the standard protocol. For immunohistochemistry, tumor sections were deparaffinized, rehydrated and heated in a microwave oven for 20 min in activity antigen retrieval solution (10 mM citric buffer, pH 6.0). Endogenous peroxidase was inactivated with 3% hydrogen peroxide solution. The slides were rinsed with distilled water, washed two times with PBS for 3 min and incubated with Superblock (Scytec Laboratories, Burlingame, CA) in a humidified chamber for 1 h at room temperature. After rinsing with PBS, the slides were incubated with avidin (Vector Laboratories, Inc., Burlingame, CA) for 15 min, washed with PBS and blocked with biotin in a humidified chamber for 15 min at room temperature. A monoclonal mouse antibody to adenovirus type 5 (Abcam, Cambridge, MA) was applied. The slides were incubated with primary antibodies overnight in humidified chambers at 4 °C. After PBS rinse, a biotinylated secondary antibody was applied to the slides and incubated for 1 h. After washing with PBS, slides were incubated with avidin-peroxidase complex reagent (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min, washed once with PBS, stained with freshly prepared diaminobenzidine solution for 15 min and counterstained with hematoxylin.

In situ terminal deoxynucleotide transferase-mediated nick end labeling assay

The *in situ* apoptosis detection kit was purchased from Roche Diagnostics. Tumor tissue sections were deparaffinized using a sequential xylene protocol and rehydrated through gradients of ethanol and distilled water. Slides were treated with 10 nmol l⁻¹ Tris solution containing 1 μ g ml⁻¹ proteinase K for 15 min. All slides were rinsed with PBS and incubated with 100 μ l terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture (or 100 μ l control labeling solution for negative control) in a humid chamber at 37 °C for 30 min. The slides were washed three times with PBS and incubated with 100 μ l TUNEL POD solution in a humid chamber at 37 °C for 30 min. After washing with PBS, the slides were stained with freshly prepared diaminobenzidine solution for 10 min, rinsed with PBS and counterstained with hematoxylin.

Results

Construction of a shorter form of PSES, M6

We initially constructed an HSV-TK armed PRRA by replacing the CMV-GFP expression cassette from AdE4PSESE1a³¹ with a PSES-HSV-TK expression cassette. However, the virus did not propagate well in 911E4 cells, limiting its clinical utility (Ahn M *et al.*, unpublished data). We believe that the problem was the packaging size limitation of the adenovirus. We modified the PSES sequence by deleting the non-functioning sequences, thereby shortening the total insert size.³⁰ Our linker mutation study indicated that L2 and L5 likely did not have a function (Figure 1a). In addition, the activity of a 90-bp upstream region in AREc3 of PSME del2 was mediated by AP-3 (Figure 1b).³² Utilizing this information, we decided to make a shorter form of PSES, called M6, by deleting the L2 and L5 sequence of AREc3 and replacing the 90-bp upstream sequence of PSME del2 with a simple AP-3 binding sequence. Figure 1c demonstrates that M6 retained tissue-specific activity in PSA and PSMA-positive cells. Replacing the PSES sequence with M6 sequence resulted in a 106 bp decrease and normal propagation in 911E4.

Construction of a TK-armed PRRA

AdIU1 was constructed by replacing the CMV-GFP expression cassette in AdE4PSESE1a³¹ with a M6-HSV-TK expression cassette to extend the therapeutic potential of the PSES-based PRRA (Figure 2). HER 911E4 cells were transfected with recombinant adenoviral cosmid linearized by Pac I restriction enzyme digestion, and AdIU1 was propagated in HER 911 E4 cells as described in Materials and methods. CsCl-purified AdIU1 was titered with the Adeno-XTM rapid titer kit and expressed as infectious units (IFU). To assess the prostate specificity and viral replication efficiency of AdIU1, an *in vitro* viral replication assay was performed. PSA/PSMA-positive and -negative cells were infected with a dose of AdIU1 different from adenovirus's similar infectivity.³¹ AdIU1 replicated as efficiently as AdE4PSESE1a in PSA/PSMA-positive C4-2 and CWR22rv cells (Table 1). The replication was diminished three- to fourfold in PSA/PSMA-negative PC-3 and DU-145. Demonstrating the fact that AdIU1 replication was tightly controlled by PSES and restricted to PSA/PSMA-positive cells.

Western blotting analysis of adenovirus E1a and herpes simplex virus thymidine kinase proteins expression

Androgen-independent, PSA/PSMA-positive CWR22rv and C4-2, as well as AI, PSA/PSMA-negative DU-145 and PC-3 were infected with standardized doses of AdIU1. Forty-eight hours after AdIU1 infection, cell lysates were collected and western blot was performed using monoclonal antibodies against Ad5E1a or polyclonal HSV1-TK antiserum. Protein expression of both Ad5E1a and HSV1-TK was detected following AdIU1-infected, PSA/PSMA-positive CWR22rv and C4-2 cells. On the other hand, the expression of Ad5E1a and HSV1-TK proteins, in AdIU1 infected PSA/PSMA-negative PC-3 and DU-145 cells were low or undetectable (Figure 3). This result indicated that PSES and M6 promoter retained its prostate specificity to mediated E1a and HSV-TK expression in AI, PSA/PSMA-positive cells.

Selective cell killing activity of AdIU1 plus GCV against AI, PSA/PSMA-positive human prostate cancer cells in vitro

An *in vitro* GCV sensitivity assay was performed. Each cell line, CWR22rv, C4-2 and DU-145 were seeded in triplicate in 24-well plates at a density of 1×10^5 or 2×10^4 cells per well and were incubated with increasing concentrations of GCV (0 – $100 \mu\text{g ml}^{-1}$). Cell viability

Table 1 Tissue/tumor-specific replication ability of AdIU1^a

Cell lines	Input doses ^b (IFU)	Output viral doses ^c (LD ₅₀) ^d	
		AdIU1	AdE4PSESE1a
C 4-2	6.6×10^4	10^6	10^6
CWR22rv	2×10^4	10^6	10^6
PC 3	2.3×10^5	10^2	10^2
DU-145	1.6×10^5	5×10^2	5×10^2

^aCells were seeded and infected with AdE4PSESE1a or AdIU-1, and the supernatants were harvested for titer assay as described in 'Materials and methods'.

^bInput viral doses mean the virus doses used to infect cells (IFU).

^cOutput viral doses mean the virus doses produced and used for LD₅₀ assay.

^dThe virus production was expressed as an LD₅₀ value (the dilution factor that caused a CPE in at least 4 wells of cells in a row of 8 wells on a 96-well plate on day 7).

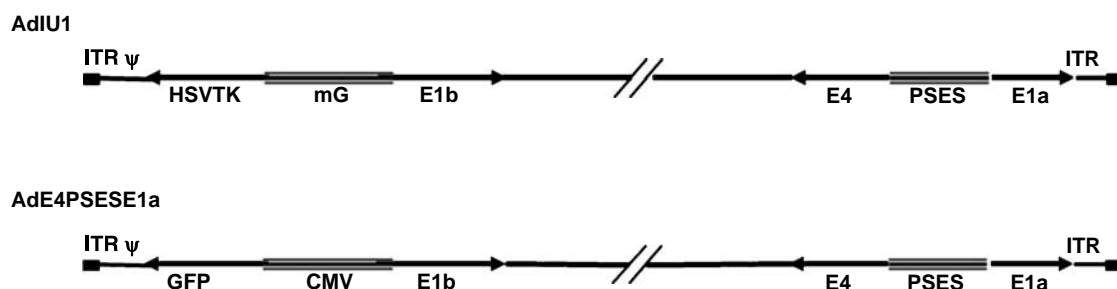


Figure 2 Schematic illustration of AdIU1. AdIU1 was constructed by placing adenoviral E1a and E4 genes under the control of PSES to direct adenovirus replication, and HSV-TK gene, a pro-drug enzyme gene, under the control of mG enhancer to maximize cell-killing activity through a bystander effect.

was determined after 5 days using crystal violet assay, and a corresponding IC_{50} dose was determined for each cell line (data not shown). An optimal non-toxic GCV treatment dose was determined to be $10 \mu\text{g ml}^{-1}$.

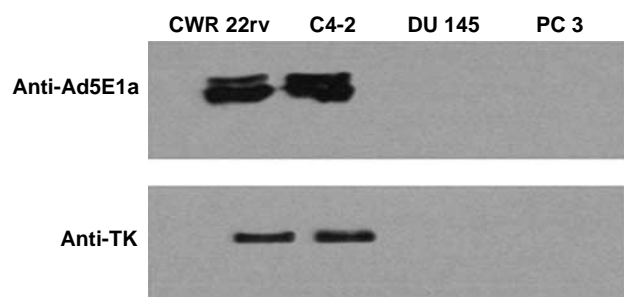


Figure 3 Expression of Ad5 E1a and HSV1-TK proteins by AdIU1 was evaluated in different cell lines. CWR22rv, C 4-2, PC-3 and DU-145 were infected with AdIU1. Forty-eight hours after viral infection, cell lysates were collected and western blot was performed. A large amount of E1a proteins ranging in size from approximately 35–46 kDa and HSV-TK (40–50 kDa) were detected in AdIU1-infected CWR22rv and C 4-2 cells. AdIU1-infected DU-145 and PC-3 did not express a detectable amount of E1a and HSV-TK proteins.

To evaluate the selective cytotoxicity of AdIU1 and AdE4PSESE1a viruses, we infected each cell line with wide dose ranges (0.0002–2 IFU per cell) of virus, and then treated infected cells with or without GCV ($10 \mu\text{g ml}^{-1}$) (Figure 4a). The growth of AI, PSA/PSMA-positive human prostate cancer cell line, CWR22rv was significantly inhibited by 0.0002 IFU of AdIU1 in the presence of GCV. AdIU1 without GCV had similar killing activity as AdE4PSESE1a either in the presence or absence of GCV. The growth of the AI, AR and PSA/PSMA-negative cell line, DU-145 was unaffected by either virus with or without GCV. In a time course experiment, CWR22rv and DU-145 cells were seeded in 24-well plates. The 24 wells were divided into four groups, no treatment, AdIU1, GCV alone and AdIU1 plus GCV treatment. The GCV alone group demonstrated limited cytotoxicity confirming that GCV ($10 \mu\text{g ml}^{-1}$) was not toxic to either of the prostate cancer cell lines. The CWR22rv cell line demonstrated cell growth inhibition at day 7 after AdIU1 exposure. The killing activity was significantly enhanced when GCV was administered following AdIU1 infection (Figure 4b). The DU-145 cell line demonstrated limited cytotoxicity in all four treatment regimens (Figure 4c).

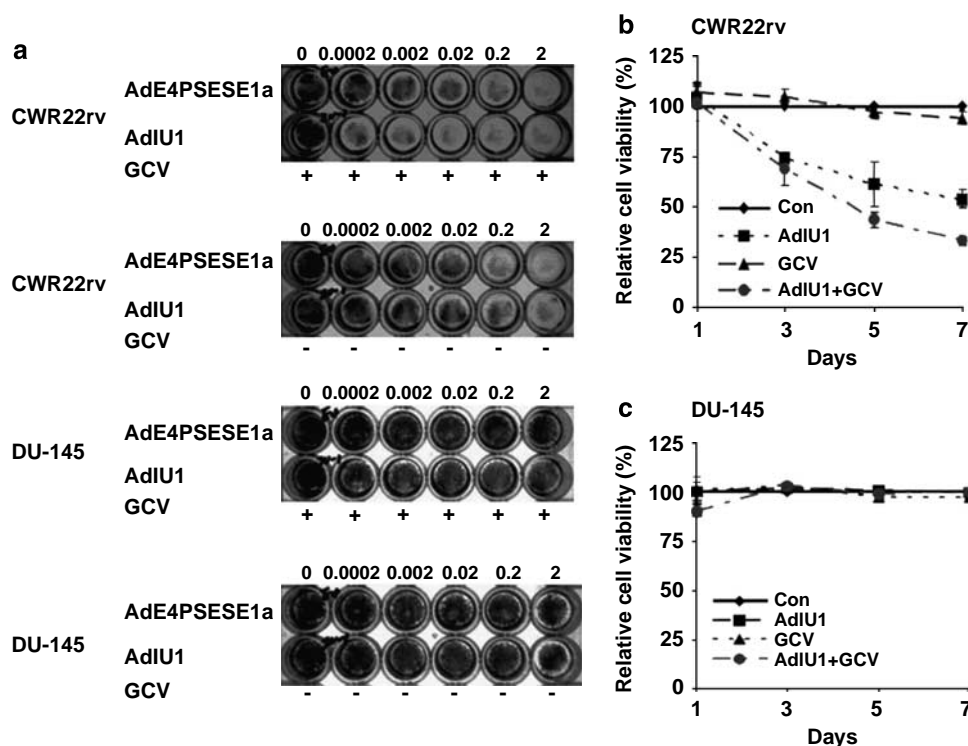


Figure 4 Dose- or time-dependent *in vitro* killing assay. 1.5×10^5 CWR22rv and DU-145 cells were seeded in 24-well plates, infected by serial dilutions of AdIU1 from 0.0002 to 2 IFU per cell, with replicative-deficient adenovirus AdE1aPSESE4 as a control, and then treated with or without GCV ($10 \mu\text{g ml}^{-1}$). Seven days after infection, cells were stained with crystal violet (a). CWR 22rv and DU-145 were treated with 0.2 IFU per cell of AdIU1 (■), $10 \mu\text{g ml}^{-1}$ of GCV (▲) or AdIU1 plus GCV (●). A group of cells without treatment (◆) were used as a control. At days 1, 3, 5 and 7, crystal violet staining was performed to detect attached cells. Then 1% SDS was added to lyse the cells and for OD590 reading. Cell survival rate curves were drawn to evaluate the killing activity of AdIU1. The growth of AdIU1-infected cells was significantly inhibited by the addition of ganciclovir (GCV) ($10 \mu\text{g ml}^{-1}$), especially in CWR22rv (b). On the other hand, the growth of DU-145 (c) was not inhibited by AdIU1 plus GCV treatment.

In vivo growth inhibition of CWR22rv xenograft by AdIU1/GCV

Human prostate CWR22rv4 xenograft tumors were induced by s.c. injection of CWR22rv cells into the flanks

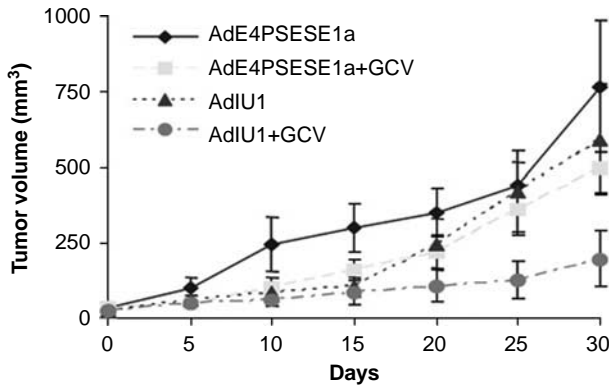
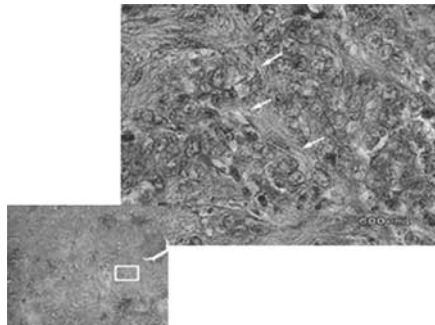


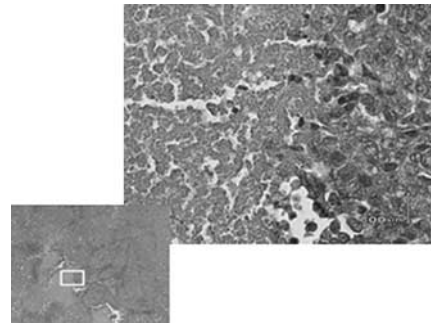
Figure 5 Tumor growth inhibition by AdIU1 plus GCV in a xenograft prostate model. CWR22rv prostate tumor xenografts were established subcutaneous in athymic nude mice. Tumors were treated with AdE4PSESE1a (◆, $n=7$), AdE4PSESE1a plus/ GCV (■, $n=8$), AdIU1 (▲, $n=8$) or AdIU1 plus GCV (●, $n=8$). Viruses were delivered by intratumoral injection at day 0, and GCV (80 mg kg^{-1} of body weight day^{-1}) was administered two times a day for 10 days. Tumor volumes were measured every 5 days. Treatment with AdIU1 plus GCV significantly inhibited the growth of CWR22rv tumors compared with treatment of the AdIU1 group.

of athymic nude mice. The mice were castrated 3 days after CWR22rv inoculation to test whether AdIU1 or AdE4PSESE1a was able to eliminate AI tumors in a castrated host. After tumor formation, the mice were randomized into four treatment groups (AdIU1, AdE4PSESE1a, AdIU1 plus GCV and AdE4PSESE1a plus GCV). After randomization to groups the mice were injected intratumorally with AdIU1 or AdE4PSESE1a on day 0. On day 5, groups receiving GCV treatments were injected with GCV (40 mg kg^{-1} body weight) two times daily for 10 days. Tumor volumes were measured every 5 days (Figure 5). AdIU1/GCV effectively inhibited the growth of CWR22rv xenografts. Light microscopic observation of hematoxylin and eosin-stained tissue sections from tumors injected with AdIU1/GCV showed substantial treatment effect and a large amount of fibrosis (Figure 6) in contrast to other groups. In addition, we observed that all necrotic tumors stained positive for apoptosis by TUNEL assay (Figures 7A–D). We observed no significant difference in apoptosis between both groups at day 30. Anti-adenovirus type 5 E1a immunohistochemical staining revealed that extensive viral infection existed throughout the AdIU1, AdE4PSESE1a and AdE4PSESE1a plus GCV treatment group tumors (Figures 7e–h); however, adenovirus staining was absent in the AdIU1 plus GCV treatment group.

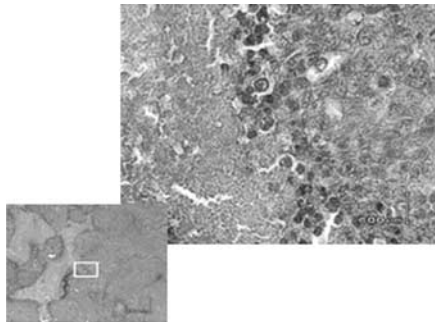
a AdIU1 + GCV



b AdIU1



c AdE4PSESE1a + GCV



d AdE4PSESE1a

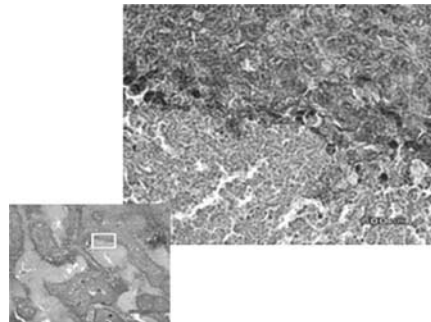


Figure 6 Histologic representations of virus-treated tumors. Tumor sections of AdIU1 plus GCV (a), AdIU1 (b), AdE4PSESE1a plus GCV (c) and AdE4PSESE1a (d) treatment groups were stained with hematoxylin and eosin. The lower left panel of each picture was taken at low power ($\times 4$ magnification), and the upper right panel is magnified to $\times 40$ (focused on white box). Tumors treated with AdIU1 plus GCV (a) demonstrated marked fibrosis and infiltration of fibroblasts (arrows). AdE4PSESE1a alone and plus GCV and AdIU1 alone treated tumors revealed healthy foci of tumor cells embedded within large areas of necrosis.

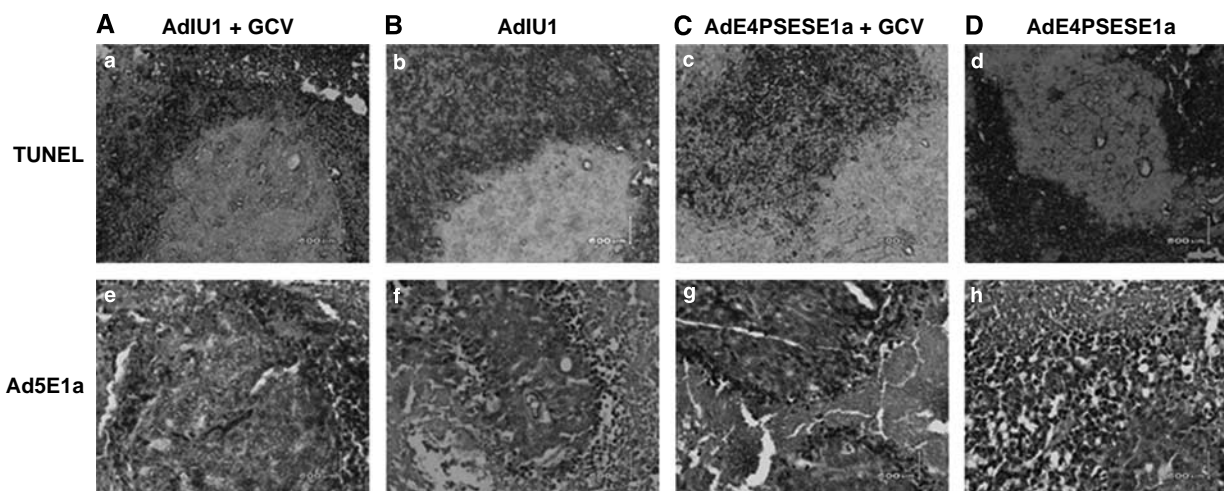


Figure 7 Apoptosis signal and Ad5E1a expression in subcutaneous tumors. All necrotic areas showed apoptotic signals by TUNEL Assay (A, AdIU1 plus GCV, B, AdIU1, C, AdE4PSESE1a plus GCV, D, AdE4PSESE1a). In the AdIU1 with GCV group, E1a expression was rare (e, AdIU1 Puls GCV, f, AdIU1, g, AdE4PSESE1a plus GCV, h, AdE4PSESE1a).

Discussion

Replication-defective recombinant adenoviruses have been widely studied *in vitro* and *in vivo* as a vector to deliver cancer therapeutic genes. Adenoviral-based cancer gene therapy still remains an unrealized potential for its ability to infect and transduce a variety of mammalian cells, including prostate cells,²⁰ in a cell cycle replication-independent manner without genotoxicity. However, there are several limitations to the use of these vectors for cancer gene therapy. To overcome deficiencies of replication-deficient recombinant adenoviruses, we have developed a pair of compact regulatory elements PSES and M6 that allow for prostate-specific regulation of several adenoviral protein and HSV-TK. The main strategy is to control the expression of adenovirus E1a genes through a tissue-specific promoter. We have enhanced the earlier strategy used to restrict adenovirus replication in hepatocellular and prostate carcinomas through α -fetoprotein and PSA promoters.^{21,33}

Gene therapy with HSV-TK as a suicide gene has been performed in a variety of tumor types *in vitro*, *in vivo* as well as in several clinical trials. Recently, Freytag *et al.*²³ described a replication competent oncolytic adenovirus delivering a suicide gene and therapy used in combination with radiotherapy.^{34–38} They demonstrated that the suicide genes CD and HSV-TK could augment the anti-tumor effects of oncolytic replication competent adenoviruses and also acts as a radiation sensitizer. However, Freytag's approach lacks prostate-specific control and excludes its use in metastatic disease. We previously showed that both PSA and OC promoters can transcriptionally regulate HSV-TK gene-based therapy to inhibit the growth of AI PSA-producing cells. Earlier, a PSA-selective replication-competent adenovirus, CG787 (or CV787) was administered intravenously to patients with

hormone-refractory metastatic prostate cancer,^{39–41} demonstrating the potential safety of systemic administration of oncolytic vectors.

The current investigation builds on the ability of the adenovirus to infect prostate cancer cells and provide both an expanded infection and a longer exogenous gene expression with a prostate-restricted replication-competent oncolytic virus, AdIU1. In previous investigations, the PSES was developed by locating the minimal sequence, AREc3 and PSME (del2) in AREc and PSME, respectively and placing AREc3 upstream from PSME (del2).³⁰ PSES showed high activity specifically in PSA/PSMA-positive and AI prostate cancer cells.³¹ L2 and L5 in AREc3 did not affect transcriptional activity and was deleted and 90-bp of proximal region of PSME was replaced by a simple AP-3 binding site. These manipulations reduced the size of PSES from 513 to 407 bp. The shortened PSES is called M6. The tissue-specific activity of M6 has been tested in several cell lines by luciferase assay showing that M6 retains strong prostate specificity being active only in PSA/PSMA-positive prostate cancer LNCaP and C4-2 cells.

In this study, we investigated the gene-directed enzyme/prodrug therapeutic effect of AdIU1, a novel PRRA expressing the M6 promoter-driven HSV-TK suicide gene. AdIU1 replicates within infected cells and kills by direct viral lysis, resulting in *in vivo* amplification of input viral dose. Additionally, AdIU1-infected cells produce HSV-TK to enhance killing by pro-drug administration.⁴² Using HSV-TK/GCV the 'bystander effect' is maximized by spreading to adjacent cancer cells after lysis of initially infected cells. Morris *et al.*⁴⁴ and Lambright *et al.*⁴³ reported that there was no advantage of adding HSV-TK/GCV therapy to a replication competent adenovirus. The result of our study suggested that AdIU1 with GCV had a better therapeutic effect than AdIU1 alone. In our animal experiment, GCV was injected 5 days after AdIU1

administration, allowing time for viral replication in the tumor. In both Morris and Lambright's papers, GCV was given right after virus administration. Further, Freytag's group demonstrated that HSV-TK/GCV therapy inhibited viral replication, so the therapeutic effect of combining replicating adenoviral vector and HSV-TK/GCV therapy is balanced between killing by viral replication and by HSV-TK/GCV. We believe that the major reason why Morris *et al.* and Lambright *et al.* did not see an advantage in adding HSV-TK/GCV therapy in a replicating adenovirus is because of their therapy regimen. GCV given to the animal immediately after virus injection inhibited viral replication, so the advantage of oncolysis was traded in for HSV-TK/GCV effect, resulting in a similar therapeutic effect between HSV-TK expressing replicating virus therapy with and without GCV. The *in vitro* tissue-specific cytotoxicity of AdIU1/GCV in CWR22rv, C4-2 and DU-145 cells was assessed. Although the growth of AI, PSA/PSMA-positive prostate cancer cell lines CWR22rv and C4-2 were significantly inhibited by a small number of AdIU1 virus particles and GCV, the growth of the AI, PSA/PSMA-negative prostate cancer cell line, DU-145 could only be inhibited by a much greater exposure to AdIU1/GCV. More importantly, the *in vivo* ability of intratumoral injection AdIU1/GCV to effectively inhibit growth of CWR22rv tumors in nude mice was confirmed. Histological analysis revealed a large number of fibroblasts infiltrating only AdIU1/GCV-treated tumors. Virus persistency was detected in AdIU1, AdE4PSESE1a and AdE4PSESE1a/GCV-treated tumors, but not in AdIU1/GCV-treated tumors. This observation is consistent with Freytag *et al.* that TK/GCV treatment inhibits adenoviral replication and suggests effective HSV-TK-mediated killing of infected and unlysed cells. In addition, it is likely that AdIU1/GCV combined with radiation therapy could have even a greater growth inhibition.

In conclusion, we have developed a prostate-restricted replicative, HSV-TK-armed adenovirus, AdIU1. AdIU1 demonstrated prostate cancer-specific-killing activity, which could be enhanced by GCV administration. Gene therapy as monotherapy against prostate cancer currently remains in its infancy. Although preventive strategies are being entertained, the ultimate clinical use of gene therapy for improving prostate cancer treatment would most likely be in combination with surgery, radiation or chemotherapy. The ability of providing prostate-specific cell killing and gene expression provides an opportunity to attempt systemic therapy with AdIU1. AdIU1 will be the focus of a phase I clinical trial for locally advanced and metastatic prostate cancer.

Acknowledgements

We thank M Black, Department of pharmaceutical Sciences, Washington State University for providing the TK-polyclonal antibody. These studies were supported by NIH K08 CA079544-01A2 and DOD DAMD 17-03-1-0077.

References

- 1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C *et al.* Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106–130.
- 2 Cheon J, Kim HK, Moon DG, Yoon DK, Cho JH, Koh SK. Adenovirus-mediated suicide-gene therapy using the herpes simplex virus thymidine kinase gene in cell and animal models of human prostate cancer: changes in tumour cell proliferative activity. *BJU Int* 2000; **85**: 759–766.
- 3 van der Poel HG, McCadden J, Verhaegh GW, Kruszewski M, Ferrer F, Schalken JA *et al.* A novel method for the determination of basal gene expression of tissue-specific promoters: an analysis of prostate-specific promoters. *Cancer Gene Ther* 2001; **8**: 927–935.
- 4 Latham JP, Searle PF, Mautner V, James ND. Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res* 2000; **60**: 334–341.
- 5 Steiner MS, Gingrich JR. Gene therapy for prostate cancer: where are we now? *J Urol* 2000; **164**: 1121–1136.
- 6 O'Keefe DS, Uchida A, Bacich DJ, Watt FB, Martorana A, Molloy PL *et al.* Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate* 2000; **45**: 149–157.
- 7 Xing Y, Lu G, Xiao Y, Zeng F, Zhang Q, Xiong P *et al.* Bystander effect mediated by herpes simplex virus-thymidine kinase/ganciclovir approach on prostatic cancer cells and its regulation. *Zhonghua Yi Xue Za Zhi* 2002; **82**: 1484–1487.
- 8 Park HS, Cheon J, Cho HY, Ko YH, Bae JH, Moon DG *et al.* *In vivo* characterization of a prostate-specific antigen promoter-based suicide gene therapy for the treatment of benign prostatic hyperplasia. *Gene Therapy* 2003; **10**: 1129–1134.
- 9 Ko SC, Cheon J, Kao C, Gotoh A, Shirakawa T, Sikes RA *et al.* Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res* 1996; **56**: 4614–4619.
- 10 Springer CJ, Niculescu-Duvaz I. Prodrug-activating systems in suicide gene therapy. *J Clin Invest* 2000; **105**: 1161–1167.
- 11 Denny WA. Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy). *J Biomed Biotechnol* 2003; **2003**: 48–70.
- 12 Schepelmann S, Springer CJ. Viral vectors for gene-directed enzyme prodrug therapy. *Curr Gene Ther* 2006; **6**: 647–670.
- 13 Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res* 1999; **59**: 1498–1504.
- 14 Matsubara S, Wada Y, Gardner TA, Egawa M, Park MS, Hsieh CL *et al.* A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res* 2001; **61**: 6012–6019.
- 15 DeWeese TL, van der Poel H, Li S, Mikhak B, Drew R, Goemann M *et al.* A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res* 2001; **61**: 7464–7472.
- 16 Sadeghi H, Hitt MM. Transcriptionally targeted adenovirus vectors. *Curr Gene Ther* 2005; **5**: 411–427.
- 17 Wu L, Matherly J, Smallwood A, Adams JY, Billick E, Belldgrun A *et al.* Chimeric PSA enhancers exhibit

- augmented activity in prostate cancer gene therapy vectors. *Gene Therapy* 2001; **8**: 1416–1426.
- 18 Diamandis EP, Yousef GM. Human tissue kallikreins: a family of new cancer biomarkers. *Clin Chem* 2002; **48**: 1198–1205.
 - 19 Hsieh CL, Gardner TA, Miao L, Balian G, Chung LW. Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. *Cancer Gene Ther* 2004; **11**: 148–155.
 - 20 Kubo H, Gardner TA, Wada Y, Koenenman KS, Gotoh A, Yang L *et al*. Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* 2003; **14**: 227–241.
 - 21 Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 1997; **57**: 2559–2563.
 - 22 Galanis E, Vile R, Russell SJ. Delivery systems intended for *in vivo* gene therapy of cancer: targeting and replication competent viral vectors. *Crit Rev Oncol Hematol* 2001; **38**: 177–192.
 - 23 Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther* 1998; **9**: 1323–1333.
 - 24 Freytag SO, Khil M, Stricker H, Peabody J, Menon M, DePeralta-Venturina M *et al*. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 2002; **62**: 4968–4976.
 - 25 Freytag SO, Stricker H, Pegg J, Paielli D, Pradhan DG, Peabody J *et al*. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res* 2003; **63**: 7497–7506.
 - 26 Edwards SJ, Dix BR, Myers CJ, Dobson-Le D, Huschtscha L, Hibma M *et al*. Evidence that replication of the antitumor adenovirus ONYX-015 is not controlled by the p53 and p14(ARF) tumor suppressor genes. *J Virol* 2002; **76**: 12483–12490.
 - 27 Rothmann T, Hengstermann A, Whitaker NJ, Scheffner M, zur Hausen H. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J Virol* 1998; **72**: 9470–9478.
 - 28 O'Shea CC, Johnson L, Bagus B, Choi S, Nicholas C, Shen A *et al*. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell* 2004; **6**: 611–623.
 - 29 O'Shea CC, Soria C, Bagus B, McCormick F. Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell* 2005; **8**: 61–74.
 - 30 Lee SJ, Kim HS, Yu R, Lee K, Gardner TA, Jung C *et al*. Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol Ther* 2002; **6**: 415–421.
 - 31 Li X, Zhang YP, Kim HS, Bae KH, Stantz KM, Lee SJ *et al*. Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer. *Cancer Res* 2005; **65**: 1941–1951.
 - 32 Lee SJ, Lee K, Yang X, Jung C, Gardner T, Kim HS *et al*. NFATc1 with AP-3 site binding specificity mediates gene expression of prostate-specific-membrane-antigen. *J Mol Biol* 2003; **330**: 749–760.
 - 33 Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J *et al*. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther* 1999; **10**: 1721–1733.
 - 34 Kim JH, Kim SH, Brown SL, Freytag SO. Selective enhancement by an antiviral agent of the radiation-induced cell killing of human glioma cells transduced with HSV-tk gene. *Cancer Res* 1994; **54**: 6053–6056.
 - 35 Kim JH, Kim SH, Kolozyvary A, Brown SL, Kim OB, Freytag SO. Selective enhancement of radiation response of herpes simplex virus thymidine kinase transduced 9L gliosarcoma cells *in vitro* and *in vivo* by antiviral agents. *Int J Radiat Oncol Biol Phys* 1995; **33**: 861–868.
 - 36 Rogulski KR, Wing MS, Paielli DL, Gilbert JD, Kim JH, Freytag SO. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. *Hum Gene Ther* 2000; **11**: 67–76.
 - 37 Rogulski KR, Freytag SO, Zhang K, Gilbert JD, Paielli DL, Kim JH *et al*. *In vivo* antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. *Cancer Res* 2000; **60**: 1193–1196.
 - 38 Freytag SO, Paielli D, Wing M, Rogulski K, Brown S, Kolozyvary A *et al*. Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. *Int J Radiat Oncol Biol Phys* 2002; **54**: 873–885.
 - 39 Small EJ, Carducci MA, Burke JM, Rodriguez R, Fong L, van Ummersen L *et al*. A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther* 2006; **14**: 107–117.
 - 40 Dilley J, Reddy S, Ko D, Nguyen N, Rojas G, Working P *et al*. Oncolytic adenovirus CG7870 in combination with radiation demonstrates synergistic enhancements of antitumor efficacy without loss of specificity. *Cancer Gene Ther* 2005; **12**: 715–722.
 - 41 Chen Y, DeWeese T, Dilley J, Zhang Y, Li Y, Ramesh N *et al*. CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. *Cancer Res* 2001; **61**: 5453–5460.
 - 42 Wang JQ, Zheng QH, Fei X, Mock BH, Hutchins GD. Novel radiosynthesis of PET HSV-tk gene reporter probes [18F]FHPG and [18F]FHBG employing dual Sep-Pak SPE techniques. *Bioorg Med Chem Lett* 2003; **13**: 3933–3938.
 - 43 Lambright ES, Amin K, Wiewrodt R, Force SD, Lanuti M, Probert KJ *et al*. Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. *Gene Therapy* 2001; **8**: 946–953.
 - 44 Morris JC, Wildner O. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. *Mol Ther* 2000; **1**: 56–62.

Introduction

This Stage I Laboratory-Clinical Transition Award proposal provides the DOD with the opportunity fund from preclinical testing level to cGMP production of several novel therapeutics against advanced prostate cancer. Support of this pipeline of novel therapeutics should lead to three initial trial and then subsequent combination clinical trial with conventional treatment options and each other. A prominent lead agent has been identified. The lead agent Ad-IU1 has resulted from ten years of investigation and collaboration of several of the core Co-investigators. The DOD provided the spark which initiated this pipeline concept by awarding a prior Clinic Trial Development Award to the PI. During the CTDA the lead agent require further refinement and this time has allow the PI to assemble a team of basic and clinical investigators focused on development of prostate cancer therapeutics. Drs. Gardner, Kao and Ko have over ten year collaborative effort in the development of novel targeted therapeutics. In our initial four years of collaborative effort, we were able to design, produce, validated, test pharmacodynamics, perform toxicology, summit an IND application and conduct a Phase I Clinical Trial of an replication defective adenovirus Ad-OC-TK (1) (See attached paper). This tumor-specific replication defective adenovirus is under investigation with our collaborators in Japan (2).

Background for Proposal: The funding of this Laboratory-Clinical Transition Award proposal affords the DOD to capitalize on this prior experience and support the development of several vectors targeting several clinical scenarios facing men with prostate cancer on a daily basis. The prior collaborative work while at the University of Virginia was the first gene transfer protocol for that institution in 1999. Indiana University has taken a lead role gene therapy and vector development and continues to be supportive environment to pre-clinically develop novel therapeutic vector towards the clinic. The Clinical Trial Working Group has formed to promote the transfer of novel therapeutic for the bench to the bedside and is the foundation of the for this application. The additional support of this proposal and the development of the lead agent, AD-IU1, through toxicology to cGMP production run will open the pipeline for several lead agents to follow.

Lead Agent(s): This proposal will focus on the development of three prostate restricted replicative adenoviral vectors capable of potential targeting benign prostate, early localized, locally advanced and metastatic prostate cancer. Using the (<http://www.cancer.gov/images/trwg/Developmental-Pathway-Agent-Drug-Biologics.pdf>) as a guideline. Ad-IU1, the lead agent (see Data to follow) is at the level of initial toxicology study. Several remaining steps along the pathway have been worked out for similar therapeutics. **Ad-IU1** is a PRRA with several of the critical adenoviral early replication proteins under the control of the strong and highly prostate specific, PSES regulatory element. AdIU1 also contains the hsv-TK gene which can allow for additional prostate specific cytotoxic effect and the possibility to image the adenoviral infection (3). **AD-IU2** is a PRRA using the PSES regulatory element to drive replication and TRAIL expression (See Data to Follow). The data demonstrates the additive or synergistic ability of this AdIU2 with radiation therapy and it development is at the dosimetry stage in combination with radiotherapy. **AdIU3** is a PRRA using PSES to regulate viral replication and the osteocalcin promoter (OC) to control hsv-TK. AdIU3 has is earliest in development and is at the initial in vivo efficacy testing on bone metastases model.

Objective:

- 1: AdIU1 GLP Toxicology KAO Study preparation of cGMP production and IND year one Gardner Trial in men with mets followed by neoadjuvant pre surgery Gardner year one
- 2: AdIU2 GLP Toxicology Study KAO and completion of dosimetry KO c GMP production and IND KO/gardner first months to 18 months Clinical trial year two KO

3: AdIU3 Completion of in vivo testing KAO year 1 toxicology and cGMP production and trial by year 3

The investigator will not wait for stage 2 funding prior to initiating cGMP production

Specific Aim 1. Pre-clinical trial testing of Ad-IU1, a prostate-specific replication-competent adenovirus (PSRCA) armed with HSV-TK expressed under control of the prostate-specific PSES promoter. Our laboratory has previously constructed a PSRCA which effectively kills androgen-independent (AI) prostate cancer cells *in vitro* and *in vivo* when administered in combination with gancyclovir (GCV). To prepare for clinical trial, Dr. Gardner will lead the laboratory to perform toxicology and safety studies with a clinical grade vector produced by the National Gene Vector Laboratory within year 1 of this proposal. The toxicology study will be designed after discussing route of administration with the Food and Drug Administration. **The main goal of this aim is to prepare Ad-IU1 for IND approval and administration in a Phase I clinical trial.**

Specific Aim 2. To perform *in vivo* dosimetry experiments on nude mouse prostate cancer xenografts and pre-clinical toxicology testing of Ad-IU2, a PSRCA armed with TRAIL under control of the prostate-specific PSES promoter. We have previously demonstrated that the neoadjuvant treatment of high risk prostate cancer with a PSRCA expressing TRAIL resulted in the radio-sensitization of tumor cells, thereby enhancing the therapeutic effect of external beam radiation therapy. The goals of this multi-modal approach combining viral lysis, apoptosis-inducing gene therapy, and radiation therapy are to achieve complete local tumor control, reduce radiation dose and associated treatment morbidities, and improve the clinical outcome for patients with high risk locally advanced prostate cancer. Dr. Ko will guide the laboratory to complete the combinatorial *in vivo* experiments and toxicology studies by year 2 of this proposal. **The main goal of this specific aim is to prepare Ad-IU2 for IND approval and translation to a Phase I clinical trial.**

Specific Aim 3. Pre-clinical evaluation of Ad-IU3, the prostate-specific replication-competent adenovirus armed with HSV-TK under control of the OC promoter. To enhance the viral transduction efficiency of our previous clinical trial virus, Ad-OC-TK, we placed the prostate epithelium- and bone stroma-targeting OC-TK expression cassette in a PSES-controlled replicative adenovirus. Dr. Kao will lead the laboratory to test its *in vitro* tumor killing activity, *in vivo* anti-tumor activity and its safety and toxicology.

Preliminary Studies

1). Ad-IU1, a TK-armed prostate-restricted replicative adenovirus

a). PSES is a prostate-specific promoter for prostate cancer gene therapy.

Through an extensive understanding of the regulation of PSA and PSMA gene expression in AI PCa, we developed PSES, a chimeric enhancer which retains high prostate-specificity regardless of androgen-status. In a transient reporter assay experiment, AREc3 showed no transcriptional activity in the absence of androgen, whereas PSME(del2) demonstrated a four- to five-fold increase in activity over PSME. PSES demonstrated significantly stronger transcriptional activity than either AREc3 or PSME(del2) in the presence or absence of androgen (Figure 1A). PSES demonstrated high prostate-specificity as seen in Figure 1B, with transcriptional activation observed only in PSA/PSMA-positive cell lines, LNCaP, C4-2 and CWR22rv. As seen in Figure 1C, PSES had similar activity as CMV promoter and five-fold higher activity than RSV promoter. To test whether PSES retained prostate-specificity in adenoviral vectors, we constructed a replication-deficient recombinant adenovirus, Ad-PSES-Luc, carrying luciferase under the control of PSES. Ad-PSES-Luc demonstrated much higher activity in PSA/PSMA-positive prostate cancer cells than in PSA/PSMA-negative cancer cells (results similar to Figure 1B). To demonstrate the ability of systemic injection of adenovirus to target prostate cancer, we established LNCaP subcutaneous tumors in athymic nude mice and injected, via tail vein, Ad-CMV-Luc or Ad-PSES-Luc. The animals were sacrificed, and the organs were harvested 2 days after viral administration for luciferase assay. As depicted in Figure 1D, both Ad-PSES-Luc and Ad-CMV-Luc demonstrated high activity in the tumor; however, as expected, activity in kidney, liver, lungs and spleen was equivalent to background levels only for Ad-PSES-Luc. Similar results were obtained with intraprostatic injection of both viruses. Due to its small size, high level of tissue specificity, and strong promoter activity in the presence or absence of androgen, PSES is an ideal promoter for use in prostate cancer gene therapy.

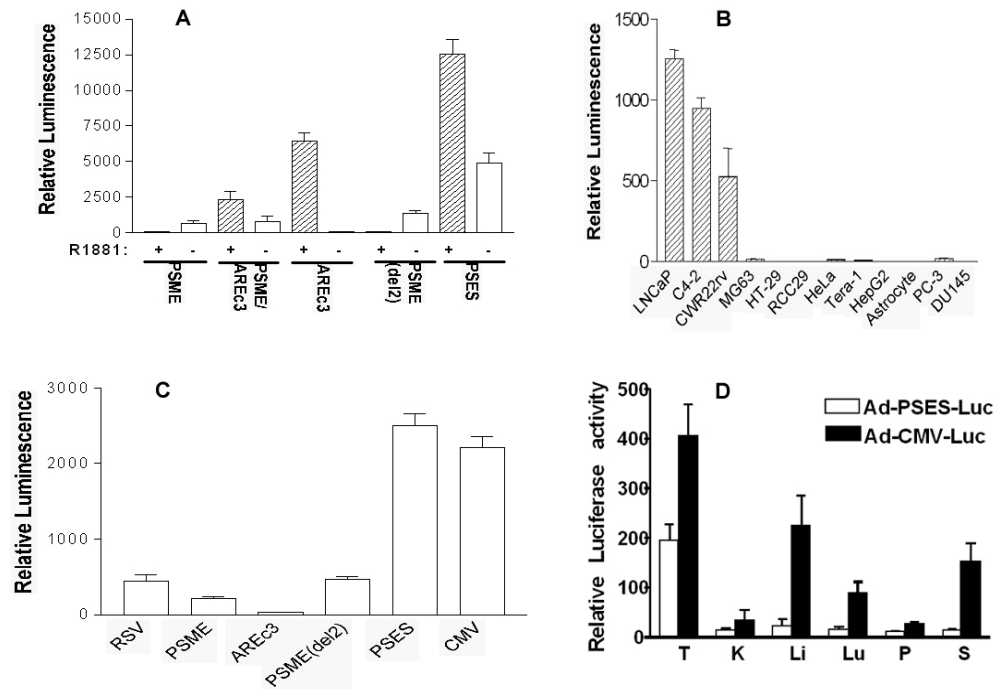


Figure 1. *PSES had strong promoter activity and high tissue-specificity.* *A*, 2×10^5 LNCaP cells seeded in 12-well plates were cotransfected with 0.5 μ g of pGL3/TATA containing PSME, PSME/AREc3, AREc3, PSME(del2) or PSES and 1 ng pRL-SV40. 3 nM androgen (R1881) added to media 5 hr after transfection. Results representative of 3 transfections. *B*, Various cells were transfected with pGL3/PSES/TATA, as described in *1A*, in the absence of androgen. LNCaP, C4-2 and CWR22rv are PSA/PSMA-positive PCa cell lines. *C*, PSES activity was compared to RSV and CMV promoters in LNCaP cells in the absence of androgen. Luciferase activity is representative of three assays. *D*, Subcutaneous LNCaP tumors induced in athymic nude mice. 7×10^{10} virus particles of Ad-CMV-Luc (■) or Ad-PSES-Luc (□) were injected via tail vein. After two days, mice were sacrificed, and organs were harvested for luciferase assay.

To test whether PSES retained prostate-specificity in adenoviral vectors, we constructed a replication-deficient recombinant adenovirus, Ad-PSES-Luc, carrying luciferase under the control of PSES. Ad-PSES-Luc demonstrated much higher activity in PSA/PSMA-positive prostate cancer cells than in PSA/PSMA-negative cancer cells (results similar to Figure 1B). To demonstrate the ability of systemic injection of adenovirus to target prostate cancer, we established LNCaP subcutaneous tumors in athymic nude mice and injected, via tail vein, Ad-CMV-Luc or Ad-PSES-Luc. The animals were sacrificed, and the organs were harvested 2 days after viral administration for luciferase assay. As depicted in Figure 1D, both Ad-PSES-Luc and Ad-CMV-Luc demonstrated high activity in the tumor; however, as expected, activity in kidney, liver, lungs and spleen was equivalent to background levels only for Ad-PSES-Luc. Similar results were obtained with intraprostatic injection of both viruses. Due to its small size, high level of tissue specificity, and strong promoter activity in the presence or absence of androgen, PSES is an ideal promoter for use in prostate cancer gene therapy.

b). Ad-E4PSESE1a is a prostate-specific replication-competent adenovirus (PSRCA).

Ad-E4PSESE1a was constructed by placing PSES and the E1a gene segment in the E4 region of the recombinant adenoviral genome. A CMV-EGFP (enhanced green fluorescent protein) cassette was inserted



Figure 2. *Helper cell transfection and generation of a PSRCA.* A, Viral genome was released from cloning vector by PacI digestion and transfected into HER911E4 cells. Stable expression of E4 by 911E4 cells allows propagation of the virus and expression of EGFP. B, AdE4PSESE1a is not propagated in native HER911 cells. C, Viral supernatant harvested from HER911E4 cells and caused lytic infection of AI PSA/PSMA-positive C4-2 cells. EGFP expression indicates viral infection.

in C4-2 cells (Figure 2C). To test the tissue-specificity of AdE4PSESE1a, various cell lines, both PSA/PSMA-positive and negative, were infected with the virus and monitored for five days after infection. As depicted in Figure 3, the number and intensity of green cells increased through day five in PSA/PSMA-positive PCa cells (CWR22rv and C4-2), but not in PSA/PSMA-negative cell lines. In addition, cytopathic effects, as evidenced by viral plaques under light microscopy were observed only in PSA/PSMA-positive cells. Together, these data suggest that AdE4PSESE1a will only replicate when E4 is provided by the cell or when its expression is transactivated by PSES in PSA/PSMA-positive PCa cells, thereby overcoming the leaky replication of E1a-controlled PSRCA and enhancing the virus' safety profile.

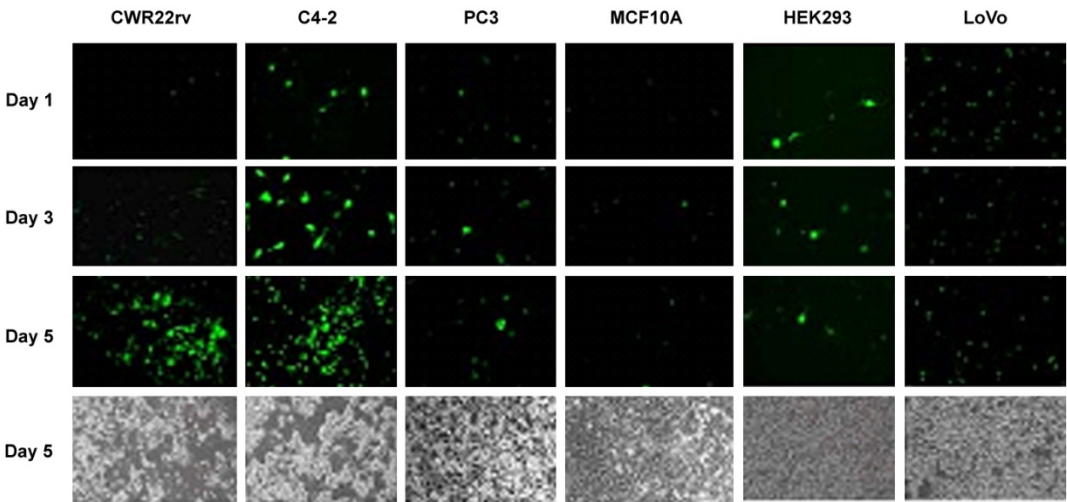


Figure 3. *PSA/PSMA-positive cells monitored daily under fluorescent microscopy after infection with AdE4PSESE1a.* EGFP expression represented viral infection and replication. At day 1 after infection, all cells expressed EGFP at low levels, regardless of PSA/PSMA status. By day 5, EGFP expression was elevated in intensity and number in PSA/PSMA-positive CWR22rv and C4-2 cells, indicating that AdE4PSESE1a had propagated and infected new cells. Bottom row, Light microscopy reveals plaques indicative of the cytopathic effect of the replicating virus, evident only in PSA/PSMA-positive cells.

c). Construction and characterization of Ad-IU1

Enzyme pro-drug suicide gene therapy has been hindered by inefficient gene delivery and transduction. To further explore the potential of this approach, we have developed Ad-IU1, a prostate-restricted replicative adenovirus (PRRA) armed with the herpes simplex virus thymidine kinase (HSV-TK). This suicide gene (HSV-TK) and pro-drug (ganciclovir (GCV)) combination has been extensively explored in both preclinical and clinical studies. We have demonstrated that Ad-IU1 effectively inhibits the growth of androgen-independent (AI) PSA/PSMA-positive prostate cancer cells both *in vitro* and *in vivo*. In this study, we aim to prepare Ad-IU1 for clinical testing. Ad-IU1 was first constructed by replacing the CMV-GFP expression cassette in Ad-E4PSESE1a (4) with a PSES-HSV-TK expression cassette. However, the virus did not propagate well in HER911E4 cells, and we believed this to be an issue with insert size during viral DNA packaging. To

circumvent this, we modified the PSES sequence by deleting two non-essential sequences within AREc3 (5), L2 and L5 which were determined to have no function within. Also, because the promoter activity in the 90 bp upstream region of AREc3 of the prostate-specific membrane enhancer (PSME) was mediated by AP-3 binding, we replaced this sequence with a simple AP-3 binding sequence. The resulting prostate-specific sequence was shortened by 1/3. As depicted in Figure 4, m6 retained tissue-specificity in PSA/PSMA-positive cells, similar to that of PSES.

Ad-IU1 was constructed by replacing the CMV-GFP expression cassette in Ad-E4PSESE1a (4) with an *m6*-HSV-TK expression cassette to enhance the therapeutic efficacy of the PSES-based PRRA. HSV-TK and *E1b* in the left arm was placed under the transcriptional control of *m6*, and *E4* and *E1a* in the right arm were placed under control of the PSES promoter. Figure 5 illustrates the structure of each virus used in this study. The adenoviral genome was released from the cloning vector by digestion with Pac I restriction enzyme and transfected into HER911E4 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The packaging cells were incubated at 37°C under 5% CO₂ for 7 to 10 days after transfection until a cytopathic effect could be observed. Ad-IU1 was further amplified in HER911E4 cells. CsCl-purified Ad-IU1 was titrated with the Adeno-XTM Rapid Titer system (BD Biosciences, Palo Alto, CA) following the manufacturer's protocol. Briefly, a dilution of the viral stock was used to infect HER 911E4 cells. 48 hrs later, these cells were fixed and stained with an antibody specific to the

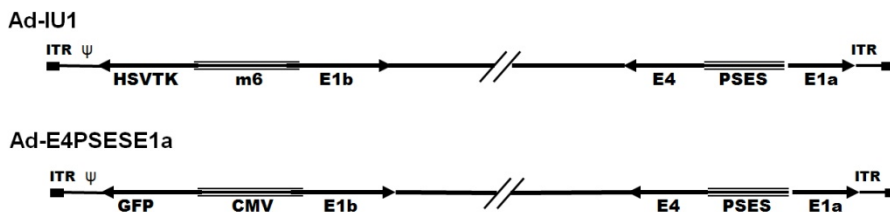


Figure 5. Genome structure of Ad-IU1 and negative control PRRA, Ad-E4PSESE1a. To construct Ad-E4PSESE1a, enhanced green fluorescent protein was cloned upstream of universal promoter, CMV into pAd1020SfidA (OD 260, Boise, ID), the adenoviral cloning vector containing the left ITR and packaging signal (Ψ), to make pAd1020SfidA-CMVEGFP, which was further digested with *SfiI* to release the left ITR and CMV-EGFP expression cassette. This fragment was cloned into pAd288E1b-E4PSESE1a. To construct Ad-IU1, the CMV-EGFP expression cassette was replaced with PSES-HSV-TK. Both adenoviral genomes were released by digestion with *PacI* and transfected into HER911E4 cells. Ad-IU1 and Ad-E4PSESE1a were further amplified in HER911E4 cells, purified by CsCl centrifugation gradient and dialyzed.

specific antigen (PSA) and osteocalcin (OC) promoters could transcriptionally regulate the HSV-TK gene in a prostate-specific manner *in vitro* and *in vivo*. This tissue-specific HSV-TK production combined with pro-drug administration inhibited the growth of AI-PSA-producing cells *in vitro*, in animal models of human prostate cancer and in patients with prostate cancer enrolled in a Phase I clinical trial of OC promoter-based HSV-TK gene therapy (1). Others have demonstrated similar *in vitro* and *in vivo* efficacy as well as safe administration to men with locally advanced and metastatic prostate cancer (6).

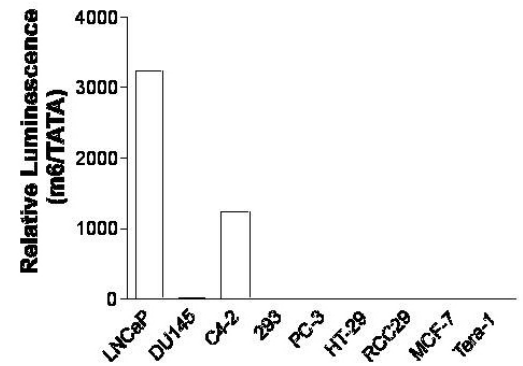


Figure 4. *m6* promoter retains similar prostate-specificity as PSES. pGL3/m6/TATA (0.5 μ g) was transfected into various cell lines (2×10^5 cells for each). After 2 days, cells were harvested, lysed with passive lysis buffer (Promega) and analyzed for luciferase activity. This experiment was conducted in the absence of androgen. Luciferase activity was standardized to transfection of cells with pGL3/TATA. pGL3/m6/TATA was active only in PSA/PSMA-positive LNCaP and C4-2 cells.

adenoviral hexon protein. Signal was detected with a secondary antibody conjugated to horseradish peroxidase (HRP). Subsequent exposure to DAB substrate turned the infected cells dark brown. Each stained cell corresponded to a single infectious unit (IFU).

Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors. Through the use of prostate-specific promoters and enhancers, the expression of a therapeutic gene or adenoviral replication can be limited to cells that contain the appropriate activators and transcription factors. We have demonstrated that both the prostate-

To assess the prostate-specificity and viral replication efficiency of Ad-IU1, an *in vitro* viral replication assay was performed. PSA/PSMA-positive and negative cells were infected with Ad-IU1 or Ad-E4PSESE1a, accounting for viral infectivity of each cell line. Once a cytopathic effect was observed, viral supernatants were collected and subjected to three freeze-thaw cycles. The viral supernatant was then used to infect HER 911E4 cells in serial dilutions in a 96 well plate. The viral replication was expressed as LD₅₀ values, the minimal dose at which 50% of cells are killed. As depicted in Table 1, Ad-IU1 replicated as efficiently as Ad-E4PSESE1a in PSA/PSMA positive C4-2 and CWR22rv cells. The replication was diminished in PSA/PSMA-negative PC-3 and DU-145 cells, demonstrating the fact that Ad-IU1 replication is tightly controlled by PSES and restricted to PSA/PSMA-positive cells.

Tissue-specific replication ability of Ad-IU1^a

Cell lines	Input Doses ^b (IFU)	Output Viral Doses ^c (LD ₅₀ ^d)	
		AdIU1	AdE4PSESE1a
C4-2	6.6×10 ⁴	10 ⁶	10 ⁶
CWR22rv	2×10 ⁴	10 ⁶	10 ⁶
PC-3	2.3×10 ⁵	10 ²	10 ²
DU145	1.6×10 ⁵	5×10 ²	5×10 ²

Table 1. *Ad-IU1 replicated efficiently in PSA/PSMA-positive cells.* 1 × 10⁶ CWR22rv, C4-2, PC-3 and DU-145 cells were plated per well in a 6-well plate and infected with the stated input dose of Ad-IU1 or Ad-E4PSESE1a, accounting for cell line infectivity. Viral supernatants were harvested 3 days after infection. HER911E4 cells were plated in 96-well plates and infected with serial dilutions of viral supernatant ranging from 1 to 10⁻¹¹, each row receiving the same dose. Cells were examined for cytopathic effect on day 7, and LD₅₀ recorded as the dose causing cytopathic effect in at least four wells in one row.

Expression of adenoviral E1a proteins and HSV-TK in PSA/PSMA-positive cells was confirmed via

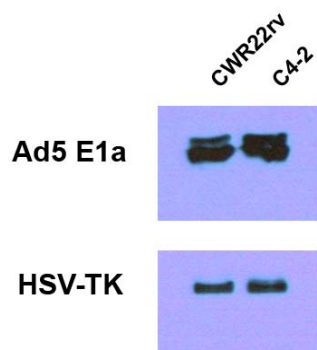


Figure 6. CWR22rv and C4-2 cells were infected with AdIU1. 48 hrs post viral infection, cell lysates were collected and Western blot was performed. E1a proteins ranging in size from approximately 35-46 kDa and HSV-TK (40-50 kDa) were detected in Ad-IU1-infected CWR22rv and C4-2 cells, but not DU-145 and PC-3 cells.

western blot analysis. AI, PSA/PSMA-positive CWR22rv and C4-2, as well as AI, PSA/PSMA-negative DU 145 and PC 3 were infected with standardized doses of Ad-IU1. 48 hrs post Ad-IU1 infection, cell lysate was collected and western blot was performed using a monoclonal antibody against Ad5 E1a or polyclonal HSV1-TK antiserum. Ad5 E1a and HSV1-TK protein expression were detected in Ad-IU1 infected, PSA/PSMA-positive CWR22rv and C4-2 cells (Figure 6); they were undetected in PSA/PSMA-negative PC3 and DU145 cells (data not shown).

d). Selective cell killing activity of Ad-IU1 in combination with GCV against PSA/PSMA-positive prostate cancer cells *in vitro*.

Herpes simplex virus *thymidine kinase* (HSV-TK)-based suicide gene therapy has been used to target prostate cancer for over a decade. The pro-drug, ganciclovir (GCV) is phosphorylated by HSV-TK to its monophosphate form, which is rapidly converted to di- and triphosphate forms by cellular kinases, the latter of which is toxic to cells. The GCV-triphosphate is incorporated into DNA during cell division, causing single-strand DNA breaks and inhibition of DNA polymerase and causes DNA chain termination, leading to cell death. An *in vitro* pro-drug sensitivity assay was performed to determine a non-toxic optimal dose for use in subsequent experiments. For this, CWR22rv, C4-2 and DU-145 were seeded in triplicate in 24-well plates and incubated with increasing concentrations of GCV (0 to 100 µg/ml). Cell viability was determined after 5 days using crystal violet assay, and a corresponding IC₅₀ dose was determined for each cell line (data not shown). The optimal non-toxic GCV treatment dose was determined to be 10 µg/ml.

To evaluate the selective cytotoxicity of Ad-IU1 and AdE4PSESE1a viruses in combination with pro-drug, we infected each cell line with wide dose ranges (0.0002 - 2 IFU/cell) of virus, and then treated infected cells with or without GCV (10 µg/ml) (Fig. 7A). The growth of the AI, PSA/PSMA-positive human prostate cancer cell line, CWR22rv, was significantly inhibited by 0.0002 IFU of Ad-IU1 in the presence of GCV vs 2 IFU

for Ad-E4PSESE1a. Ad-IU1 without GCV had similar killing activity as AdE4PSESE1a either in the presence or absence of GCV. The growth of the AI, AR- and PSA/PSMA-negative cell line, DU-145, was unaffected by either virus with or without GCV. In a time course experiment, CWR22rv and DU-145 cells were seeded in 24 well plates. The 24 wells were divided into 4 groups, no treatment, Ad-IU1, GCV alone, and Ad-IU1 plus GCV treatment. The GCV alone group demonstrated limited cytotoxicity. This confirmed that 10 $\mu\text{g/mL}$ GCV treatment was not toxic to either prostate cancer cell line. CWR22rv prostate cancer cells demonstrated cell growth inhibition at day 7 after Ad-IU1 exposure; however, the killing activity was significantly enhanced when GCV was administered following Ad-IU1 infection (Fig. 7B). DU-145 prostate cancer cells demonstrated similar survival as untreated cells following infection with Ad-IU1 in the presence or absence of GCV (Fig. 7C).

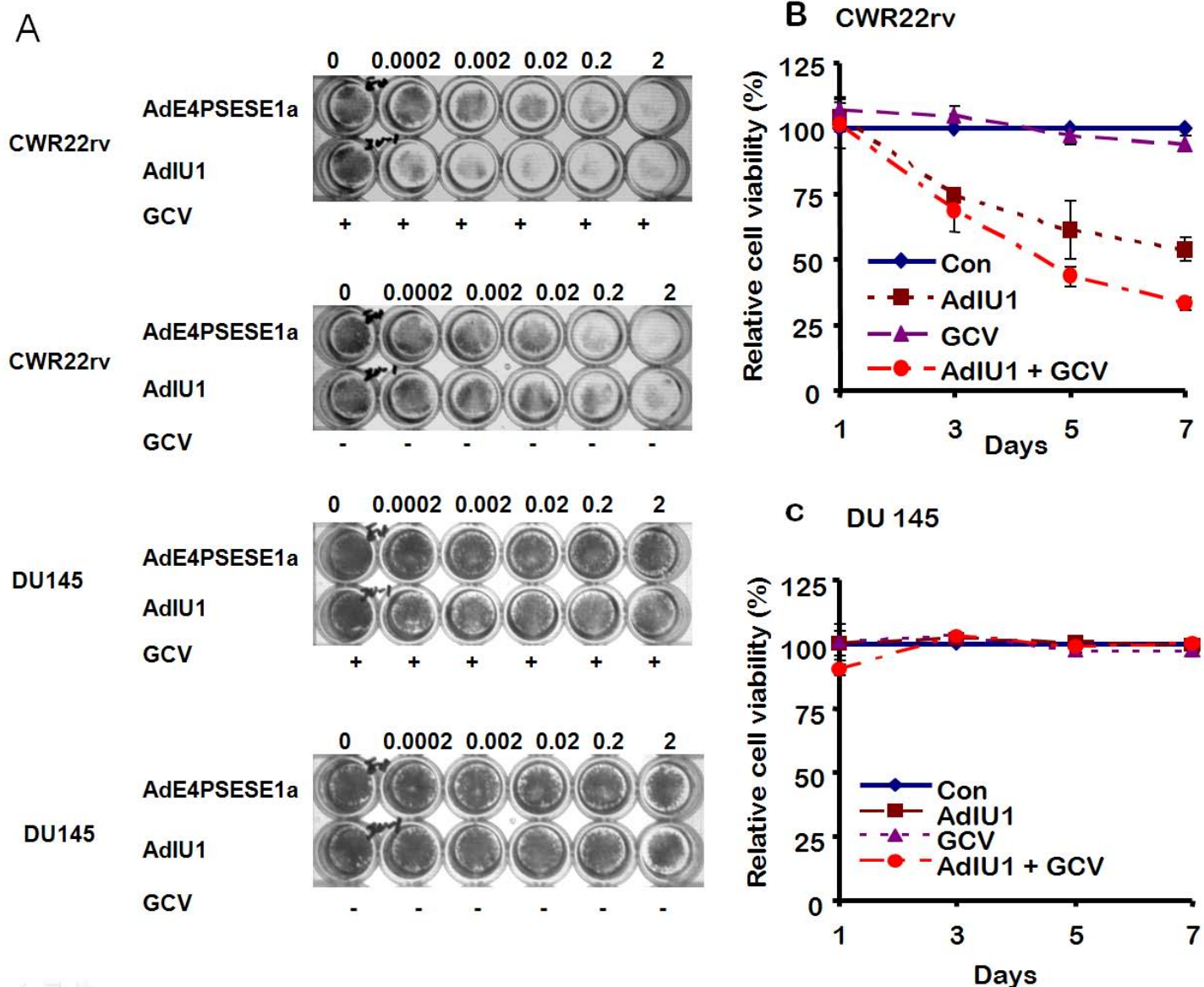


Figure 7. Dose or time-dependent *in vitro* killing assay. 1.5×10^5 CWR22rv and DU-145 cells were seeded in 24-well plates, infected by serial dilutions of AdIU1 from 0.0002 to 2 IFU/cell, with replicative-deficient adenovirus AdE1aPSESE4 as a controls, and then treated with or without GCV (10 $\mu\text{g/mL}$). Seven days after infection, cells were stained with crystal violet (A). CWR 22rv and DU 145 were treated with 0.2 IFU/cell of AdIU1 (■), 10 $\mu\text{g/mL}$ of GCV (▲) or AdIU1 plus GCV (●). A group of cells without treatment (◆) were used as a control. At days 1, 3, 5, and 7, crystal violet staining was performed to detect attached cells. Then 1% SDS was added to lyse the cells and for OD₅₉₀ reading. Cell survival rate curves were drawn to evaluate the killing activity of AdIU1. The growth of AdIU1-infected cells was significantly inhibited by the addition of Ganciclovir (GCV) (10 $\mu\text{g/mL}$), especially in CWR22rv (B). On the other hand, the growth of DU-145 (C) was not inhibited by AdIU1 plus GCV treatment.

e). In vivo growth inhibition of CWR22rv xenografts by Ad-IU1/GCV

Human prostate cancer CWR22rv xenograft tumors were induced by subcutaneous injection of CWR22rv cells into athymic nude mice. The mice were castrated 3 days after CWR22rv inoculation to test

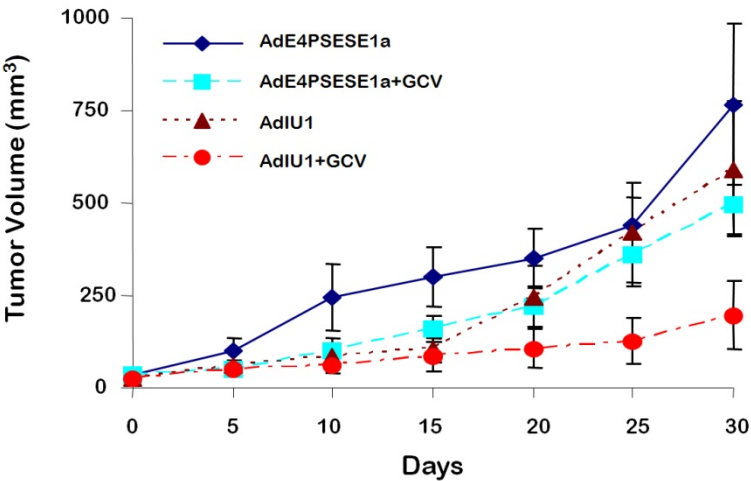


Figure 8. Tumor growth inhibition by AdIU1 plus GCV in a xenograft prostate model. CWR22rv prostate tumor xenografts were established s.c. in athymic nude mice. Tumors were treated with AdE4PSESE1a (♦, n=7), AdE4PSESE1a plus GCV (■, n=8), AdIU1 (▲, n=8) or AdIU1 plus GCV (●, n=8). Viruses were delivered by intratumoral injection at day 0 and GCV (80 mg/kg of body weight/day) was administered 2 times a day for 10 days. Tumor volumes were measured every 5 days. Treatment with AdIU1 plus GCV significantly inhibited the growth of CWR22rv tumors compared to the Ad-IU1 group.

whether Ad-IU1 or AdE4PSESE1a was able to eliminate AI tumors in a castrated host. After tumor formation, the mice were randomized into 4 treatment groups (Ad-IU1, AdE4PSESE1a, Ad-IU1 plus GCV and AdE4PSESE1a plus GCV). The mice were injected intratumorally with Ad-IU1 or AdE4PSESE1a. Day 0 was the time of virus injection. On day 5, groups receiving GCV treatments were injected with GCV (40 mg/kg body weight) 2 times a day for 10 days. Tumor volumes were measured at the times indicated in Figure 8. Ad-IU1/GCV effectively caused growth delay of CWR22rv xenografts. Light microscopic observation of H&E-stained tissue sections from tumors injected with Ad-IU1/GCV showed substantial treatment effect (Fig. 9). We observed a large amount of fibrosis following combined Ad-IU1 and GCV treatment. Additional staining for adenovirus and apoptosis were performed (data not shown). We observed that all necrotic tumors stained positive for apoptosis by TUNEL assay. We observed no significant difference in apoptosis between both groups at 30 days after injection. Anti-adenovirus type 5 E1a immunohistochemical

staining revealed that extensive viral infection existed throughout the the treatment period for the Ad-IU1, AdE4PSESE1a, and AdE4PSESE1a plus GCV treatment groups; however, adenoviral staining was absent in the Ad-IU1 plus GCV treatment group.

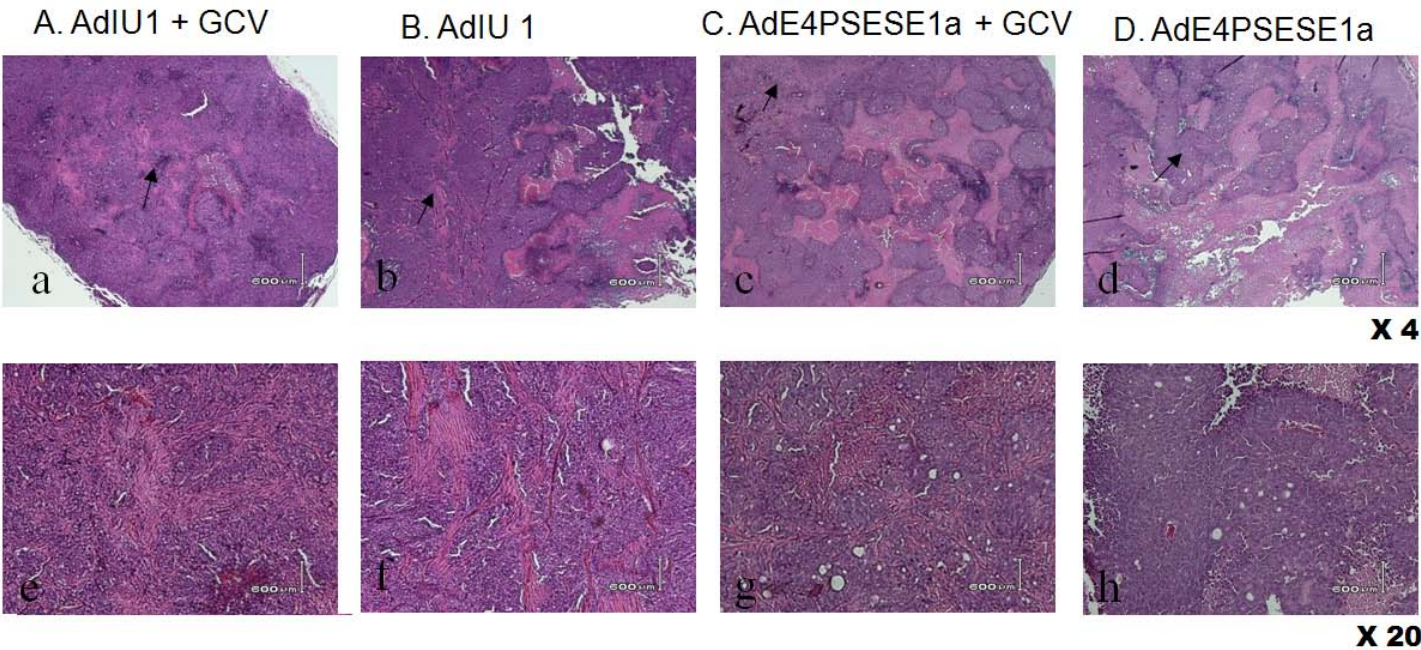


Figure 9. Histologic representations of virus-treated tumors. Tumor sections of treated tumors. Upper panel arrows indicate mass fibrosis. Lower panel shows infiltrating fibroblasts in fibrotic regions.

2). Ad-IU2, a TRAIL-armed prostate-restricted replicative adenovirus

1. Ad-IU2: a PSRCA armed with TRAIL

Recent replication-competent oncolytic viral vectors overcome the limitations of poor gene transduction efficiency observed in early clinical trials which utilized replication-deficient adenoviral vectors. In addition to the propagation of the viral vector within the tumor environment, direct cell killing by viral replication is a second advantage of oncolytic adenoviral vectors; however, despite initial growth suppression, tumors eventually fail to respond to treatment. To enhance the cell killing potential of a PSRCA, we inserted TRAIL, a potent inducer of apoptosis. To generate Ad-IU2 (Figure 10), we modified Ad-E4PSESE1a, a PSRCA in which the *E1* promoter was deleted and *E1a* was moved to the right ITR *E4* region under the control of the bidirectional PSES enhancer (4). A CMV-EGFP expression cassette in the *E1a* region was replaced with full-length, membrane-bound TRAIL cDNA under the control of PSES, just upstream of adenoviral *E1b*.

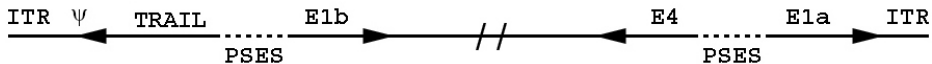


Figure 10. *Genome structure of Ad-IU2.* To construct Ad-IU2, human full-length *TRAIL* cDNA was cloned from pORF-hTRAIL (InvivoGen, San Diego, CA) downstream of PSES into pAd1020SfidA (OD 260, Boise, ID), the adenoviral cloning vector containing the left ITR and packaging signal (Ψ), to make pAd1020SfidA-PSESTRAIL, which was further digested with *SfiI* to release the left ITR and PSES-TRAIL expression cassette. This fragment was cloned into pAd288E1b-E4PSESE1a. The adenoviral genome was released by digestion with *PacI* and transfected into HER911E4 cells. Ad-IU2 was further amplified in HER911E4 cells, purified by CsCl centrifugation gradient and dialyzed.

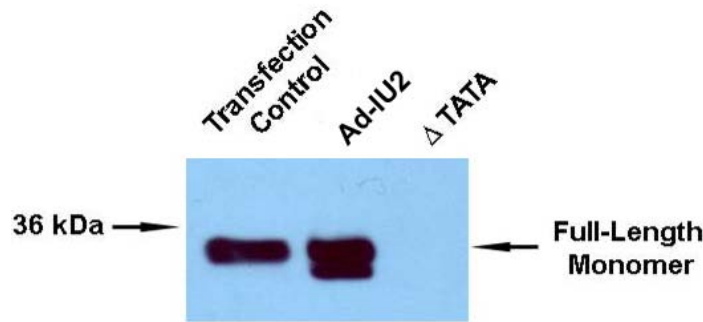


Figure 11. *TRAIL expression was confirmed in Ad-IU2-infected CWR22rv prostate cancer cells.* CWR22rv cells were infected with Ad-IU2 or Ad- Δ TATA-E1a (replication-deficient negative control) at an MOI of 1000 for 48 hours. As a positive control, cells were transfected with pORF-hTRAIL. Cell lysates were harvested with RIPA buffer, separated by 4-10% SDS-PAGE and probed with anti-TRAIL antibody (Santa Cruz).

replicated as efficiently as Ad-E4PSESE1a in PSA/PSMA-positive CWR22rv, C4-2 and LNCaP prostate cancer cell lines; however, because adenoviral early genes are not expressed in PSA/PSMA-negative cell lines such as PC-3 and DU-145, Ad-IU2 replication efficiency was comparable to that of Ad- Δ TATA-E1a in these cell lines.

Table 2. *Ad-IU2 replicated efficiently in PSA/PSMA-positive cells.* 1 x 10⁶ CWR22rv, C4-2, LNCaP, PC-3 and DU-145 cells were plated per well in a 6-well plate and infected with the stated input dose of Ad- Δ TATA-E1a, Ad-E4PSESE1a or Ad-IU2, accounting for cell line infectivity. Viral supernatants were harvested 3 days after infection. HER911E4 cells were plated in 96-well plates and infected with serial dilutions of viral supernatant ranging from 1 to 10⁻¹¹, each row receiving the same dose. Cells were examined for cytopathic effect on day 7, and LD₅₀ recorded as the dose causing cytopathic effect in at least four wells in one row.

Cell Line	Input Dose (vp)	Output Viral Dose (LD ₅₀)		
		Δ TATA	Ad-E4PSESE1a	Ad-IU2
CWR22rv	1 x 10 ⁹	3.4 x 10 ²	3.7 x 10 ⁶	3.7 x 10 ⁶
C4-2	1 x 10 ⁹	7 x 10 ¹	4 x 10 ⁵	4 x 10 ⁵
LNCaP	1 x 10 ⁹	7 x 10 ¹	7 x 10 ⁵	3.7 x 10 ⁶
PC-3	1 x 10 ¹⁰	7 x 10 ¹	4 x 10 ¹	4 x 10 ¹
DU-145	1 x 10 ¹⁰	7 x 10 ¹	7 x 10 ¹	7 x 10 ¹

b. Apoptosis induction by Ad-IU2

To test the ability of Ad-IU2 to induce apoptosis in prostate cancer cells, PSA/PSMA-positive CWR22rv, C4-2 and LNCaP cells, as well as PSA/PSMA-negative cell lines, PC-3, DU-145 and HeLa, were infected with 1000 MOI Ad-IU2, Ad-IU1 (replication-competent negative control) or mock PBS treatment. Cells were analyzed 24 hours after infection by FACS analysis for Annexin V and propidium iodide (PI) staining (Figure 12). Ad-IU1 served as the negative PSRCA control rather than Ad-E4PSESE1a, as the EGFP expressed by infected cells would read false-positive for Annexin V-FITC. Ad-IU1 was constructed in a similar fashion as Ad-IU2; however, a PSES-HSV-TK expression cassette replaces the PSES-TRAIL expression cassette. Without administration of a nucleoside analogue prodrug, the only cytotoxicity provided by Ad-IU1 is due to replication of the virus. Following Ad-IU1 infection, no significant apoptosis was induced above baseline levels for all cell lines.

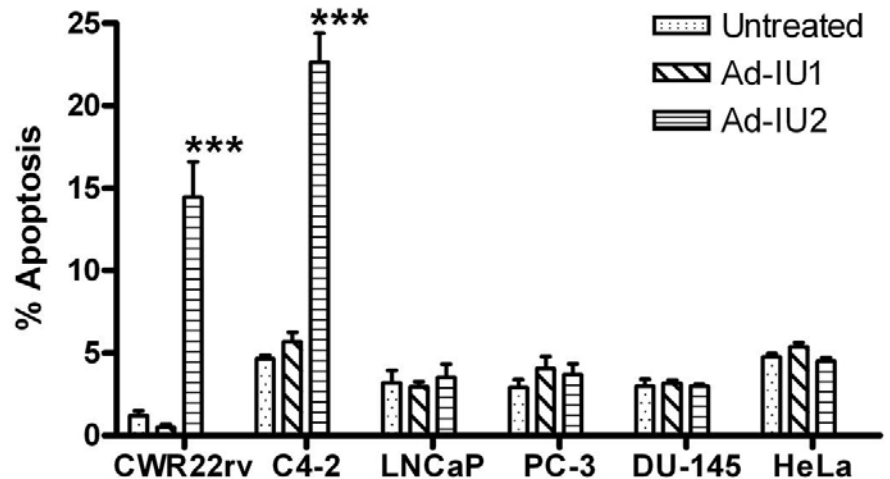


Figure 12. *Ad-IU2 induced apoptosis in PSA/PSMA-positive prostate cancer cell lines.* Cells were seeded (1×10^5 cells per well) overnight in a 24-well plate. Cells were infected with 1000 MOI Ad-IU2, Ad-IU1 or PBS mock treatment and analyzed by FACS analysis for apoptosis at 24 hours post-infection. Apoptosis was detected in PSA/PSMA-positive CWR22rv and C4-2 prostate cancer cells following Ad-IU2 infection. (***) $p < 0.001$

As host cell apoptosis occurs late in adenoviral infection, no significant apoptosis was expected in the Ad-IU1 infected group. Apoptosis following Ad-IU2 infection was induced approximately 5-fold higher in PSA/PSMA-positive CWR22rv and C4-2 cells above baseline or replication-induced apoptosis. As expected, no apoptosis above baseline level was detected in PC-3, DU-145 and HeLa cells. No apoptosis was detected in LNCaP cells, a highly TRAIL-resistant cell line (8).

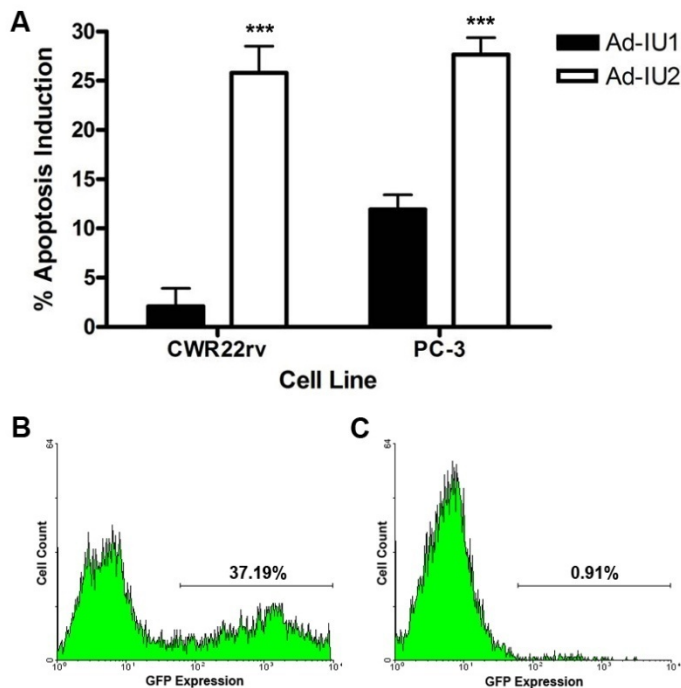


Figure 13. *Ad-IU2 bystander effect is mediated through conditioned media.* A, 1×10^6 CWR22rv cells were infected with 1000 MOI Ad-IU1 (negative control) or Ad-IU2 for 48 hours. Harvested media were heat-inactivated and applied to 1×10^5 CWR22rv cells. Apoptosis was detected by FACS analysis at 24 hours. (***) $p < 0.001$. CWR22rv cells were analyzed for GFP expression 24 hours after treatment with conditioned media from Ad-E4PSESE1a-infected CWR22rv cells without heat-inactivation (B) or with heat-inactivation (C).

c. TRAIL-mediated bystander effect

Because PSRCAs cannot achieve 100% transduction efficiency in human prostate tumors, a therapy that exerts a bystander killing effect on neighboring uninfected cells is important. Furthermore, because the PSA and PSMA profiles of cells within tumors of the prostate vary from patient to patient, such an approach enhances the therapeutic efficacy of a PSES-based PSRCA. We have demonstrated a TRAIL-mediated bystander effect in co-culture of PSA/PSMA-positive and -negative cells (data not shown), whereby interaction between infected CWR22rv and PSA/PSMA-negative PC-3 cells resulted in apoptosis induction in PC-3 cells. Although the exact method of this bystander effect is unknown, it may be mediated by cell-to-cell contact or through contact of a TRAIL receptor with TRAIL expressed on the surface of apoptotic bodies. To further investigate

the mechanism and to confirm the bystander effect, CWR22rv cells were infected with 1000 MOI Ad-IU1 or Ad-IU2. After 48 hours, media were harvested and heat-inactivated at 56°C for 30 minutes. CWR22rv or PC-3 cells were treated with the heat-inactivated media for 24 hours and analyzed by FACS analysis for apoptosis (Figure 13A). PC-3 cells treated with Ad-IU2 conditioned media underwent significant levels of apoptosis, comparable to that of similarly-treated CWR22rv cells. As shown in Figure 3, direct infection of PC-3 cells by Ad-IU2 resulted in no induction of apoptosis. To confirm that adenovirus was inactivated by thermal treatment, CWR22rv cells were infected with 1000 MOI Ad-E4PSESE1a for 48 hours, and harvested media were heat-inactivated for 30 minutes at 56°C. CWR22rv cells were treated with Ad-E4PSESE1a conditioned media for 24 hours and analyzed by FACS analysis for GFP expression, which resulted in 37.19% positivity (Figure 13B) vs. 0.91% (Figure 13C) following heat-inactivation. **In addition to enhancing the antitumor activity of Ad-IU2, a potential bystander effect would enhance the radio-sensitization properties of Ad-IU2.**

d. Antitumor activity of Ad-IU2

The *in vitro* cell killing ability of Ad-IU2 was tested in PSA- and PSMA-positive prostate cancer cell lines, CWR22rv, C4-2 and LNCaP. Cells were plated in 24-well plates and infected with varying MOIs of Ad-IU2, Ad-E4PSESE1a or Ad- Δ TATA-E1a, and media were changed 24 hours after infection. 3 days after viral infection, plates were stained with crystal violet. As depicted in Figure 14, Ad-IU2 demonstrated higher cell killing activity at lower doses than the PSRCA control, Ad-E4PSESE1a, or the replication-deficient control, Ad- Δ TATA-E1a. This significant difference in cytotoxicity was attributed to the effect of TRAIL rather than viral replication, as Ad-E4PSESE1a did not achieve similar cell killing levels even at higher MOIs. Of note, LNCaP cells were shown to be resistant to TRAIL-mediated apoptosis at 24 hours (Figure 12); however, long-term infection with Ad-IU2 resulted in effective cell killing within 3 days, even at low doses (Figure 14C). This is consistent with other reports that adenoviral infection sensitizes resistant cancer cells to TRAIL (9). Importantly, Ad-IU2 was not toxic to human dermal fibroblasts (data not shown), confirming the prostate-specificity of the vector and the tumor-selectivity of TRAIL.

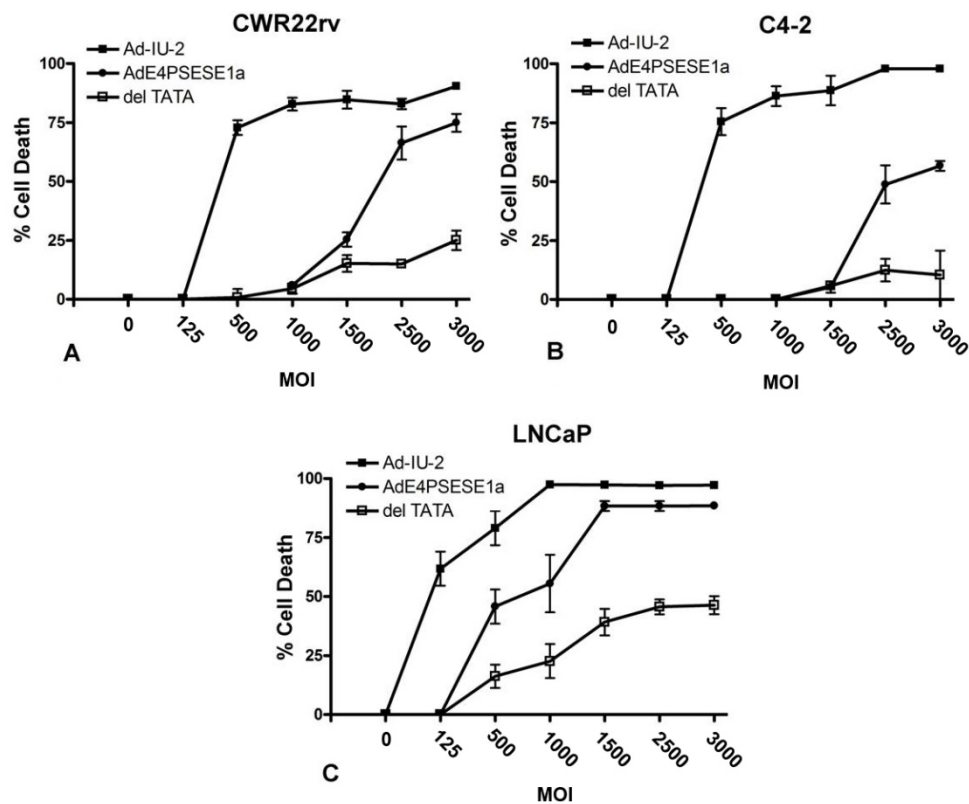
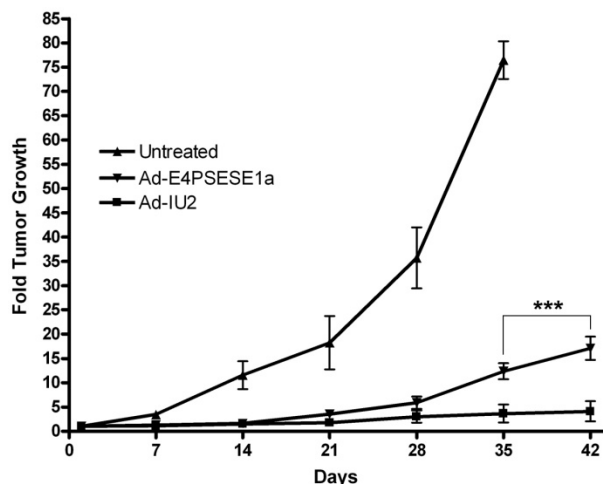


Figure 14. Ad-IU-2 demonstrated better early-killing of prostate cancer cells than a PSRCA. 1×10^5 CWR22rv, LNCaP or C4-2 cells were plated and infected with Ad- Δ TATA-E1a (negative-control replication-defective adenovirus), AdE4PSESE1a or Ad-IU-2 at the indicated doses 24 hours after plating. Plates were stained with crystal violet 3 days after infection. Early viral killing activity is represented as % cell death. Enhanced early killing effect of Ad-IU-2 above that of a PSRCA is mediated by TRAIL and not oncolysis, as only one replication cycle occurred 3 days post-infection.

To test the *in vivo* antitumor efficacy of Ad-IU2, subcutaneous CWR22rv tumor xenografts were induced by injecting 2×10^6 CWR22rv cells into the bilateral flanks of castrated, athymic mice. After 3 weeks, when palpable tumors were established, intratumoral injections of Ad-IU2, Ad-IU1 or PBS were administered. As



depicted in Figure 15, Ad-IU2 significantly suppressed the growth of CWR22rv xenografts as compared to Ad-IU1 (4.1 vs. 17.1-fold growth of tumor). Untreated mice were sacrificed at 5 weeks due to overwhelming tumor burden. Of the 9 tumors treated with Ad-IU-2, 6 responded favorably to treatment. 28 days after treatment, Ad-IU1 treated tumors began to fail therapy, whereas Ad-IU2 continued to suppress tumor growth. **These data provide evidence supporting the use of TRAIL to enhance the efficacy of an oncolytic virus against androgen-independent prostate cancer.**

Figure 15. *Ad-IU2 inhibited the growth of subcutaneous, androgen-independent CWR22rv xenografts in nude mice.* Tumors were induced by inoculating 2×10^6 CWR22rv cells s.c. on bilateral flanks of athymic mice. Once tumors were palpable (27 mm^3), they were treated with either 1×10^9 v.p. Ad-E4PSESE1a, Ad-IU2 or PBS intratumorally. Ad-IU2 suppressed the growth of CWR22rv tumors 4-fold better than Ad-IU1 (** $p < 0.001$).

e. Radio-sensitization properties of Ad-IU2

To determine the combined effect of Ad-IU2 infection and radiation treatment on prostate cancer cell lines, we performed a clonogenic survival assay. 1×10^6 CWR22rv cells were seeded in 25 cm^2 flasks and infected with 0, 150, 300 or 450 MOI Ad-IU2. 24 hours after infection, media were changed and cells were irradiated with a GammaCell (Cs-137 source) irradiator for a dose of 0, 1, 2 or 3 Gy. 24 hours after irradiation, cells were trypsinized, counted and plated at a density of 1250 cells per p100 tissue culture dish. After 3 weeks, colonies were stained with crystal violet and counted. As shown in Figure 16A, combination therapy with Ad-IU2 and RT resulted in a significant decrease in the number of colonies formed. The interaction between Ad-IU2 and RT was synergistic ($p=0.0065$), and was most significant at a viral dose of 300 MOI for all radiation doses tested (Figure 16B). **Pretreatment of cells with 300 MOI Ad-IU2 sensitized cells to radiation therapy as evidenced by a reduction in the shoulder of the radiation dose-response curve for CWR22rv cells and a reduction in the D_0 (RT dose at which 37% of cells form colonies) from 3.25 Gy without Ad-IU2 to 1.625 Gy with 300 MOI Ad-IU2.** This effect is observed when radiation-sensitizing agents reduce the ability of a cell to repair its ionization-

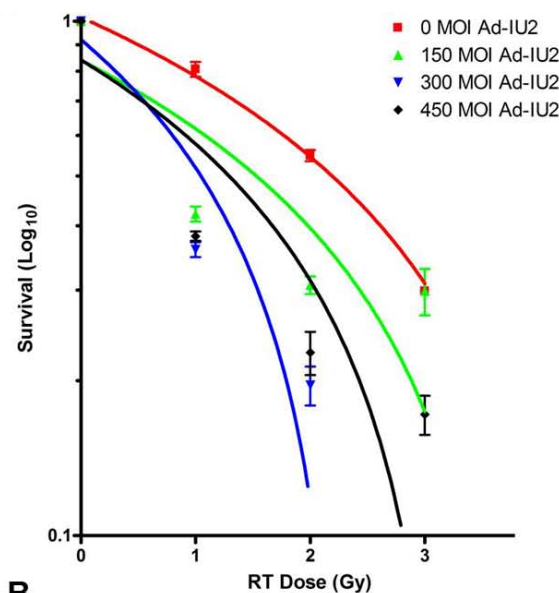
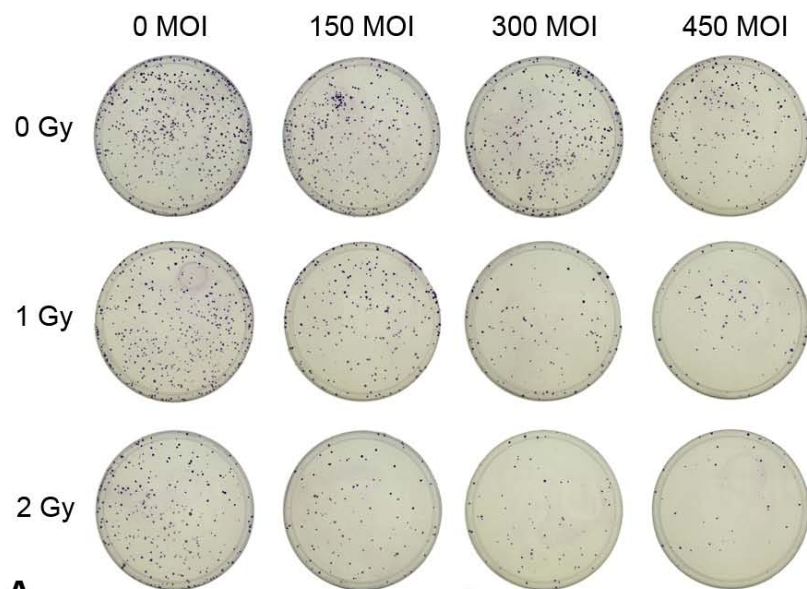


Figure 16. *Ad-IU2 sensitized CWR22rv cells to treatment with RT.* CWR22rv cells were seeded (1×10^6 cells per flask) overnight and infected with 0, 150, 300 or 450 MOI Ad-IU2. 24 hours after infection, cells were irradiated at a dose of 0, 1, 2 or 3 Gy. 24 hours following RT, treated cells were plated at densities of 1250 cells per plate and allowed to form colonies for 3 weeks. A, Plates were stained with crystal violet, and colonies (50 cells or greater) were counted. B, The dose-response survival curve indicates a synergistic interaction between RT and Ad-IU2. This effect was most pronounced at 300 MOI, where the shoulder to the dose-response curve was reduced.

induced DNA damage.

A similar effect was observed when LNCaP cells were pretreated with Ad-IU2 for 24 hours prior to RT (Figure 17). As with CWR22rv cells, the greatest sensitization occurred with an Ad-IU2 dose of 300 MOI, indicating that radio-sensitization by Ad-IU2 is not exclusive to CWR22rv. To demonstrate that radio-sensitization was attributed to TRAIL rather than adenoviral infection, we treated 1×10^6 CWR22rv cells with 0, 150, 300 or 450 MOI Ad-E4PSESE1a for 24 hours, followed by irradiation at a dose of 0, 1 or 2 Gy. 24 hours after RT, 1250 cells were plated for clonogenic survival. As depicted in Figure 18A, minimal reduction in clonogenic survival was observed with an increase in negative control virus dose. Likewise, slight reduction in colony formation was observed by treatment with radiation alone. In combination, enhancement of cell killing was not observed. **Therefore, the radio-sensitization of prostate cancer cells by Ad-IU2 is specific to TRAIL and is not attributable to adenoviral infection.** Figure 9B depicts a graphical representation of the clonogenic survival assay. No significant difference was observed between the survival curves for 0, 150 or 300 MOI; however, infection with 450 MOI Ad-E4PSESE1a resulted in 100% survival compared to the plating efficiency of unirradiated cells infected with Ad-E4PSESE1a at 450 MOI. The results from these clonogenic assays are preliminary and will be verified by the specific aims of this proposal.

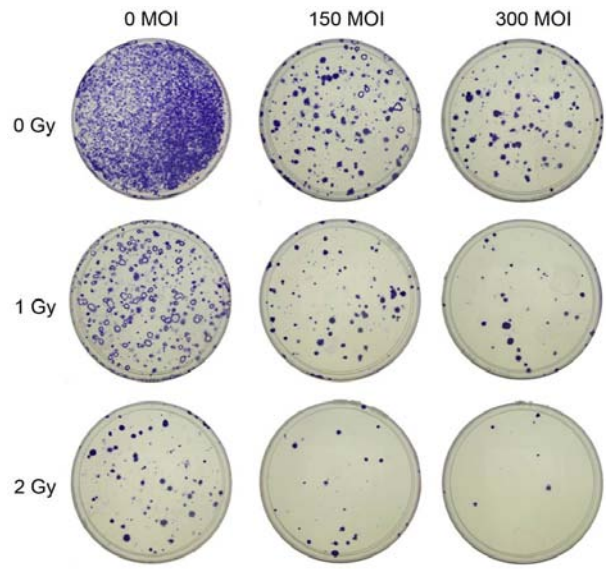
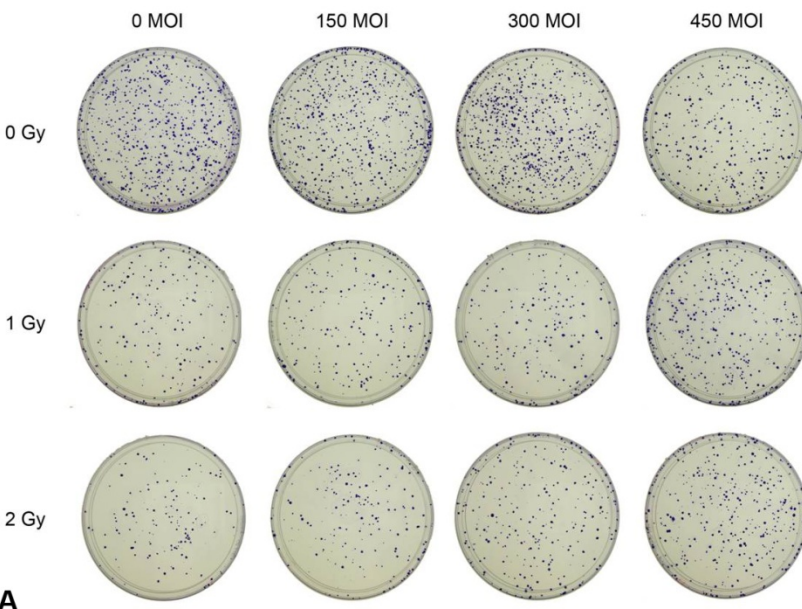


Figure 17. *Ad-IU2 sensitized LNCaP cells to treatment with RT.* 7.5×10^5 LNCaP cells were infected with 0, 150 or 300 MOI Ad-IU2 for 24 hrs and irradiated for a dose of 0, 1 or 2 Gy. 24 hours post-irradiation, cells were plated for clonogenicity at a density of 1250 cells per plate. After 2.5 weeks, cells were stained with crystal violet. Colonies were not counted because the untreated control resulted in a lawn of colonies. The greatest combined effect was observed at 2 Gy and 300 MOI Ad-IU2.

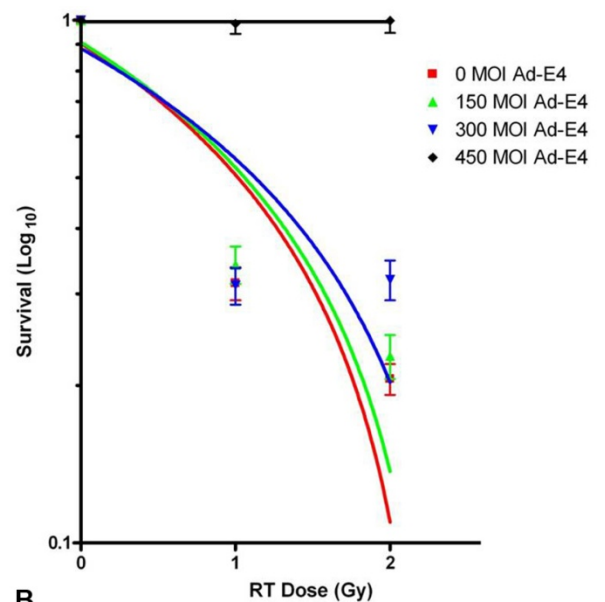


Figure 18. *Radiation-sensitization of CWR22rv cells is specific to TRAIL.* CWR22rv cells were seeded (1×10^6 cells per flask) overnight and infected with 0, 150, 300 or 450 MOI of Ad-E4PSESE1a. 24 hours after infection, cells were irradiated at a dose of 0, 1, or 2 Gy on a GammaCell Cs-137 irradiator. 24 hours after RT, treated cells were plated at densities of 1250 cells per plate and allowed to form colonies for 3 weeks. A, Plates were stained with crystal violet, and colonies (50 cells or greater) were counted. Ad-E4PSESE1a failed to enhance the effect of RT in CWR22rv cells. B, The dose-response survival curves for 0, 150 and 300 MOI Ad-E4PSESE1a-infected cells are similar, indicating no radiation-sensitization by a PSRCA alone. Of note, infection with 450 MOI Ad-E4PSESE1a enhanced the survival of irradiated CWR22rv cells.

f. Mechanism for TRAIL-mediated radiation-sensitization

To understand the mechanism responsible for the combined effect of Ad-IU2 and RT in prostate cancer cells, we studied the effect of combined treatment on apoptosis induction. 1 x 10⁵ CWR22rv cells were plated in 24-well plates and infected with 300 MOI Ad-IU2 or PBS. 24 hours after infection, media were changed and cells were irradiated for a dose of 0 or 3 Gy. These doses were selected because they achieved maximal effect in the clonogenic survival assay. 24 hours after irradiation, cells were analyzed for Annexin V and PI staining by FACS analysis. As depicted in Figure 19, at 300 MOI, Ad-IU2 failed to induce significant levels of apoptosis above the untreated control. Likewise, 3 Gy of radiation failed to induce significant levels of apoptosis as compared to the untreated control, and Ad-IU2 failed to enhance apoptosis induction when combined with radiation therapy. Previous studies have used comparable doses of virus and RT to achieve reduction of clonogenicity; however, in order to demonstrate augmentation of apoptosis following combinatorial therapy, doses far greater than clinically relevant were used. **These data suggest that at minimal doses sufficient to reduce clonogenic survival in CWR22rv cells, enhancement of apoptosis does not occur and therefore may not be a mechanism of synergy in the combined therapy.**

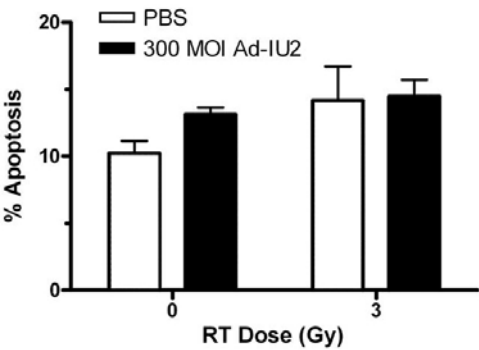


Figure 19. Combination of Ad-IU2 and radiation failed to enhance the apoptotic response in CWR22rv cells. No significant difference was observed between viral treatment with or without RT as compared to the untreated control.

Rezacova *et al* demonstrated the ability of TRAIL to progress cells through the radiation-induced G₂ phase arrest (10). To test whether this occurs in prostate cancer cells, CWR22rv cells were treated with 300 MOI Ad-IU2, Ad-E4PSESE1a or PBS. 24 hours after infection, cells were irradiated for a dose of 0 or 3 Gy. 24 hours post-irradiation, cells were permeabilized with NP-40 detergent, treated with RNase A and stained with PI. DNA content was analyzed by FACS analysis. As depicted in Figure 20, 3 Gy RT without virus infection resulted in 33.24% cells in G₂ phase. This was similar to the G₂ phase arrest of Ad-E4PSESE1a-treated cells irradiated for 3 Gy; however, CWR22rv cells pretreated with 300 MOI Ad-IU2 experienced a reduction in the G₂ phase arrest to 25.45%, similar to that of unirradiated cells treated with Ad-IU2. The reduction of G₂ phase arrest appeared to be specific to Ad-IU2 and may be attributed to TRAIL; however, adenovirus is known to perturb the cell cycle, forcing cells into S phase to allow replication of the viral DNA. For this reason, these findings must be confirmed using recombinant TRAIL. **Abrogation of the G₂ phase arrest would inhibit repair of DNA damage caused by ionizing radiation and may be a mechanism resulting in the enhancement of EBRT by Ad-IU2 infection.**

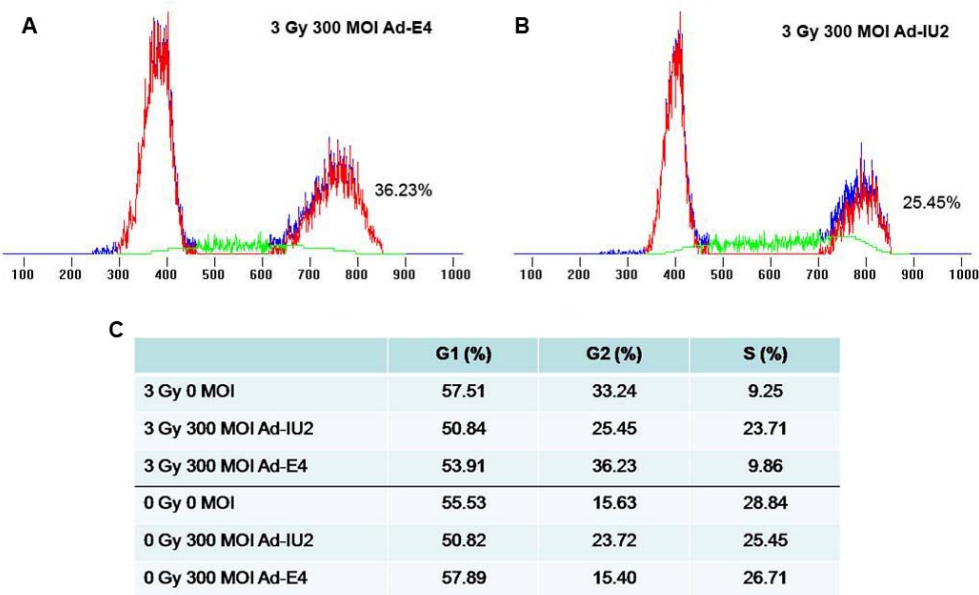


Figure 20. Pretreatment of CWR22rv cells with Ad-IU2 resulted in reduction of the radiation-induced G₂ arrest. CWR22rv cells were pretreated with 300 MOI Ad-IU2, Ad-E4PSESE1a or PBS, then irradiated for 0 or 3 Gy. DNA content was analyzed by PI staining and FACS analysis. A, 3 Gy irradiation resulted in an arrest of cells at G₂/M which was not reversed by infection with Ad-E4PSESE1a. B, Ad-IU2 infection resulted in a 29.75% reduction in the radiation-induced G₂ arrest, compared to irradiated cells infected with control virus. C, Complete cell cycle data is shown for both irradiated and unirradiated cells.

3). Ad-IU3, a PSES-driven replication-competent adenovirus armed with OC-TK

a. Osteocalcin, prostate-specific promoter for gene therapy of metastatic lesions

Osteocalcin (OC) is a highly conserved bone gamma-carboxyglutamic acid protein (BGP) that has been shown to be transcriptionally regulated by 1,25-dihydroxyvitamin D₃ (11). This noncollagenous bone protein constitutes 1-2% of the total protein in bone, and its expression is limited to differentiated osteoblasts and osteotropic tumors (12). Figure 1 depicts expression of OC RNA and protein in primary and metastatic prostate cancers. The osteoblastic nature of osseous prostate cancer metastases is well characterized (13), and the mechanism is believed to be via its osteomimetic properties, specifically its ability to express bone-related proteins such as OC (14). The human OC promoter contains numerous regulatory elements including a vitamin D-responsive element (VDRE), making it inducible by vitamin D₃ administration (15, 16), a glucocorticoid response element (GRE), an AP-1 binding site (17), and an AML-1 binding site which has been shown to be responsible for 75% of OC expression (18). The OC promoter retains its tissue specificity in a recombinant OC promoter-driven thymidine kinase (TK)-expressing adenoviral vector. Following infection with Ad-OC-TK, only cells of osteoblastic lineage expressed TK; furthermore, Ko *et al* demonstrated that the addition of acyclovir (ACV) resulted in osteoblast-specific cell toxicity (19). As depicted in Figure 21, OC is expressed greatest in metastatic prostate tumors, suggesting a role for OC to target and direct therapies to osseous metastatic lesions.

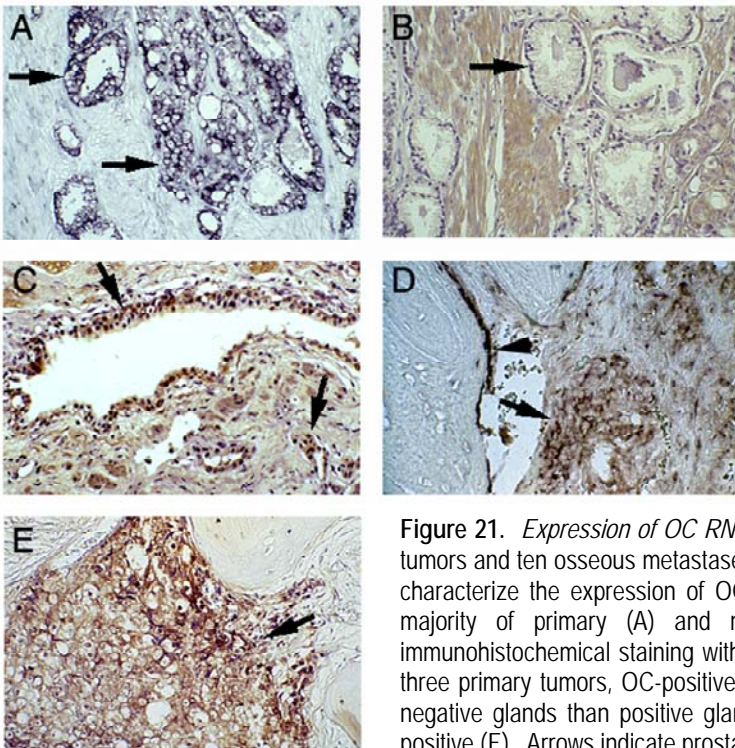
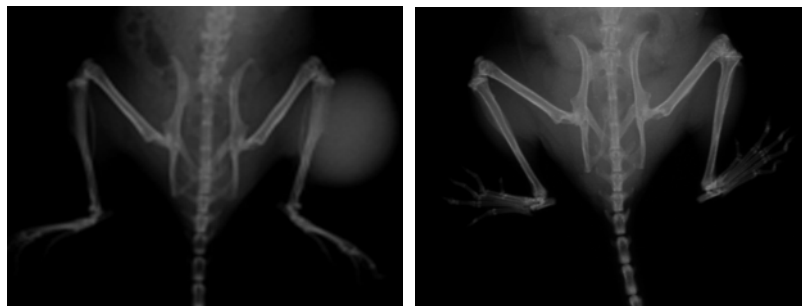


Figure 21. Expression of OC RNA and protein in primary and metastatic prostate cancers. Nine primary tumors and ten osseous metastases were stained positive for OC protein. RNA *in situ* hybridization was to characterize the expression of OC in prostate cancer cells. OC RNA transcripts were detected in the majority of primary (A) and metastatic (D) prostate cancers. OC protein was detected by immunohistochemical staining with OC4-30 anti-OC antibody (Takara Shuzo Co., LTD, Shiga, Japan). In three primary tumors, OC-positive staining was homogeneous (C); however, the other six had more OC-negative glands than positive glands (B). On the other hand, all ten osseous metastases stained OC-positive (E). Arrows indicate prostate cancer. The arrow-head indicates an osteoblast.

b. Replication-competent Ad-OC-E1a

Our laboratory developed a conditional replication-competent adenoviral vector (Ad-OC-E1a) using the mouse OC promoter **to restrict the expression of E1a to prostate epithelia and its supporting bone stroma in osseous metastases of prostate cancer.** This virus appears to be more effective than the PSE-controlled virus at killing a broader spectrum of prostate cancer cells including LNCaP, C4-2, and ARCaP (PSA-positive) as well as PC-3 and DU-145 (PSA-negative). Intratumoral injection of Ad-OC-E1a was effective at obliterating subcutaneous androgen-independent PC-3 tumors in athymic mice. In addition, intraosseous



C4-2 prostate cancer xenografts responded very well to the systemic administration of Ad-OC-E1a. 100% of the treated mice responded

Figure 22. Therapeutic effect of Ad-OC-E1a on prostate bone tumors. C4-2 prostate cancer cells were injected into the bone marrow space of the right tibia in SCID mice. The left panel shows the X-ray the untreated mouse with a large mass and deformed tibia. The right panel shows the X-ray of a mouse following intravenous administration of Ad-OC-E1a.

with a drop in the serum PSA below detectable levels. At the conclusion of the study, 40% of the treated mice were cured of prostate cancer as no PSA rebound or prostate cancer cells in the skeleton were detected (20). Figure 22 shows the X-ray of a mouse with a C4-2 bone tumor before and after treatment with Ad-OC-E1a. This study served as proof of principle that OC can drive expression in prostate epithelia and supporting bone stroma also that a replication-competent adenovirus is effective therapy for osseous metastases of prostate cancer.

It has been shown that controlling the expression of the early gene *E1b* in addition to *E1a* results in better viral replication control (21). For this reason, we developed a second replication-competent adenoviral vector, Ad-hOC-E1, containing a single bidirectional human OC promoter to control the expression of both *E1a* and *E1b*. Under the control of this VDRE-containing promoter, viral replication is induced 10-fold higher than wild-type viral replication and cytotoxicity is enhanced with the administration of vitamin D (22). Although still controversial (23), some preclinical studies indicate that vitamin D has an antiproliferative effect on androgen independent prostate cancer (24, 25). In our preclinical studies, administration of vitamin D₃ in nude mice with subcutaneous DU-145 xenografts demonstrated a therapeutic effect; however, the systemic administration of Ad-hOC-E1 in combination with vitamin D showed marked repression of the tumors, indicating the potential for clinical use (22).

c. Previous and ongoing clinical trials: replication-deficient Ad-OC-TK

Our laboratory developed a clinical protocol to test the hypothesis that the OC promoter can regulate *HSV-TK* expression specifically within a prostate cancer cell and the supportive stroma of a metastasis. We performed a phase I clinical trial enrolling eleven patients with locally recurrent or metastatic prostate cancer. Two post-surgical local recurrences and nine metastatic lesions (five osseous and 4 lymph node) were injected with replication-defective Ad-OC-TK vector followed by the administration of oral valacyclovir (26). All patients tolerated this therapy with no severe adverse effects. Of the eleven men, local cancer cell death was

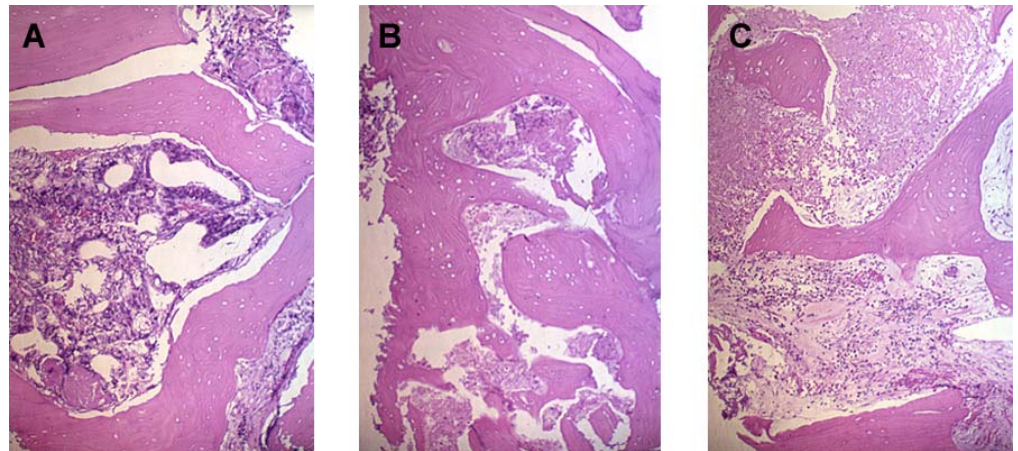


Figure 23. *Histomorphologic study of prostate cancer bone metastases treated with Ad-OC-TK.* Tissue biopsies were collected from one patient and H&E-stained before (A), during (day 8) (B), and after (day 30) (C) treatment with Ad-OC-TK and VAL. Malignant prostate cancer cells surround healthy bone tissue before treatment (A). During treatment, cancer cells are replaced and fibrosis occurs (B). By day 30, malignant cells disappear and are replaced completely by fibrosis and

observed in seven patients; however, the treated lesions of all eleven men

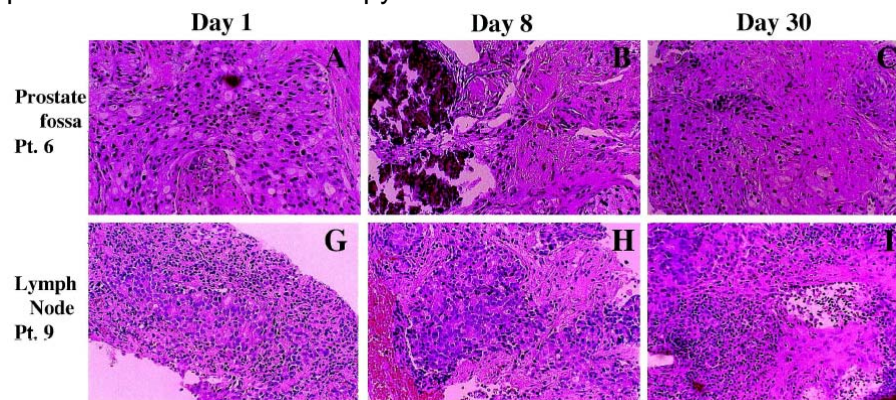


Figure 24. *Histomorphologic study of prostate cancer local recurrence and lymph node metastasis treated with Ad-OC-TK.* A, B, C correspond, respectively, to biopsy of treated prostatic fossa. At day 1 (A), tumor cells can be seen. By day 8 following treatment, inflammatory response began (B), and by day 30 (C), the local recurrent tumor was replaced by scar tissue. A similar process was observed in a treated lymph node at day 1 (D), day 8 (E) and day 30 (F).

showed histological changes as a result of the treatment. One patient demonstrated regression and stabilization of the treated lesion for 317 days post-treatment without alternative treatments, as demonstrated in Figure 23. Metastatic lesions were sampled by biopsy throughout the course of treatment to follow response to therapy. All tumors responding to therapy demonstrated an acute inflammatory response characterized by infiltration of leukocytes by day 8, tumor regression and fibrosis by day 30 (Fig. 24) (1).

Hinata *et al* has six men in Kobe, Japan for a phase I/II dose escalation study of Ad-OK-TK based on the demonstration of safety and tolerance from the previous phase I clinical trial. Currently, the low-dose treatment has been completed, and the high-dose administration is ongoing. One of three patients responded with a decrease in PSA from 318.3 ng/ml to 4.9 ng/ml. This patient received no other definitive therapy for his metastatic prostate cancer aside from hormonal therapy, and therefore, his drop in PSA was attributed to the TK gene therapy. Figure 25 depicts the course of his treatment in PSA values, where 4.9 ng/ml was achieved by day 200. On CT scan, radiographic evidence of partial tumor regression by 30 days post-treatment and complete regression by 180 days were observed (Fig. 26). This delayed response in PSA reduction and tumor regression may be attributed to the development of a cytotoxic T cell antitumor immunity (2).

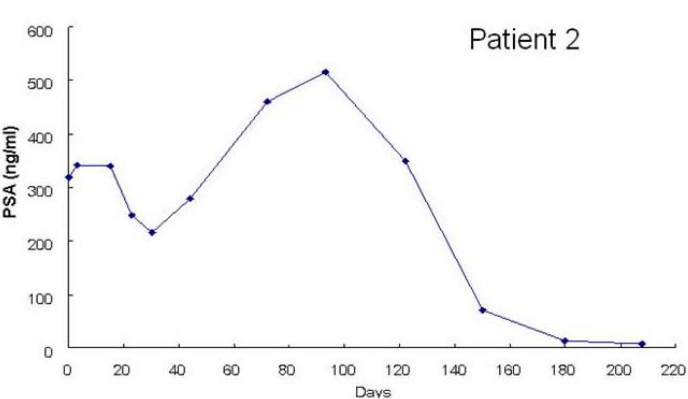


Figure 25. PSA values of patient 2. Ad-OC-TK was injected into a large, L3 bone metastasis, and PSA values were measured throughout the course of the molecular therapy. Despite an initial decrease and spike in PSA, values achieved a remarkable 4.9 ng/ml by day 200. This delayed response could be attributed to a TK-induced immune response.

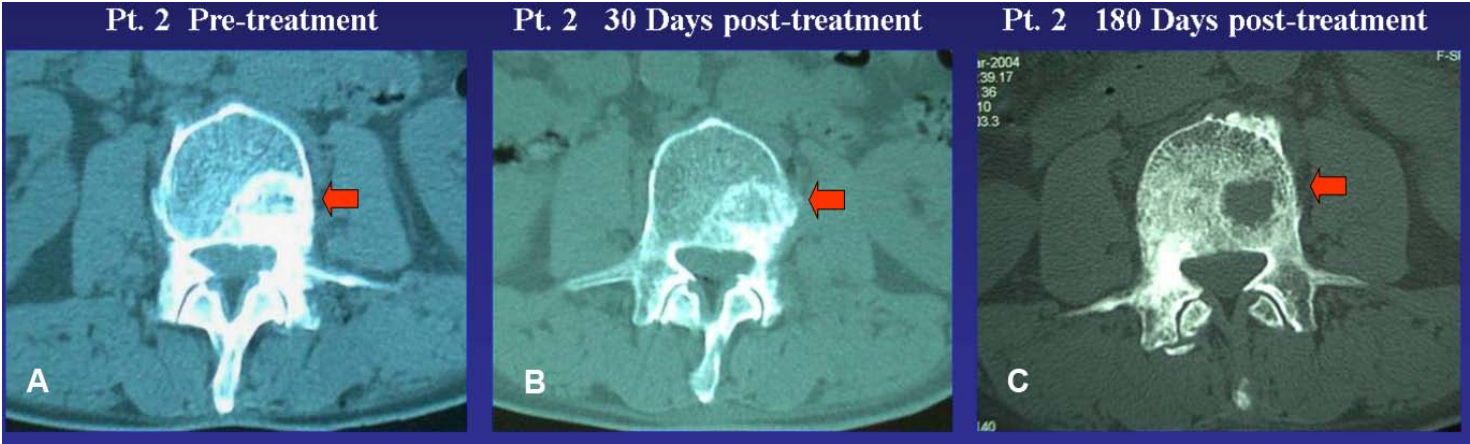
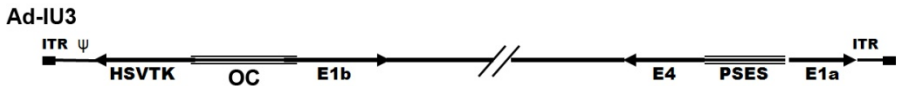


Figure 26. Radiographic evidence of tumor regression following treatment of L3 tumor with Ad-OC-TK. Patient 2 was injected intralesionally at day 1 and day 8. By day 30, partial regression was observed (B) and by day 180, complete regression of the tumor was observed (C).

This trial opened the door to the development of replication-competent adenoviral vectors for the systemic treatment of osseous and visceral prostate cancer metastases. One limitation of the previous study is poor gene transduction efficiency by replication-deficient viral vectors. Recently, we have developed a replication-competent adenovirus, Ad-IU3 in which HSV-TK expression is driven by the OC promoter; however, the expression of E1a and E4 is controlled



by the prostate-specific promoter, PSES (Fig. 27). This will improve viral killing effect and allow for systemic administration of the vector. As shown in Figure 28, Ad-IU3 effectively killed androgen independent CWR22rv cells in combination with GCV. The osteosarcoma cell line, MG63 was also tested; however, because replication is dependent on PSA and PSMA expression, Ad-IU3 did not replicate in MG63 cells, thereby demonstrating a less dramatic cytotoxic effect (Figure 28).

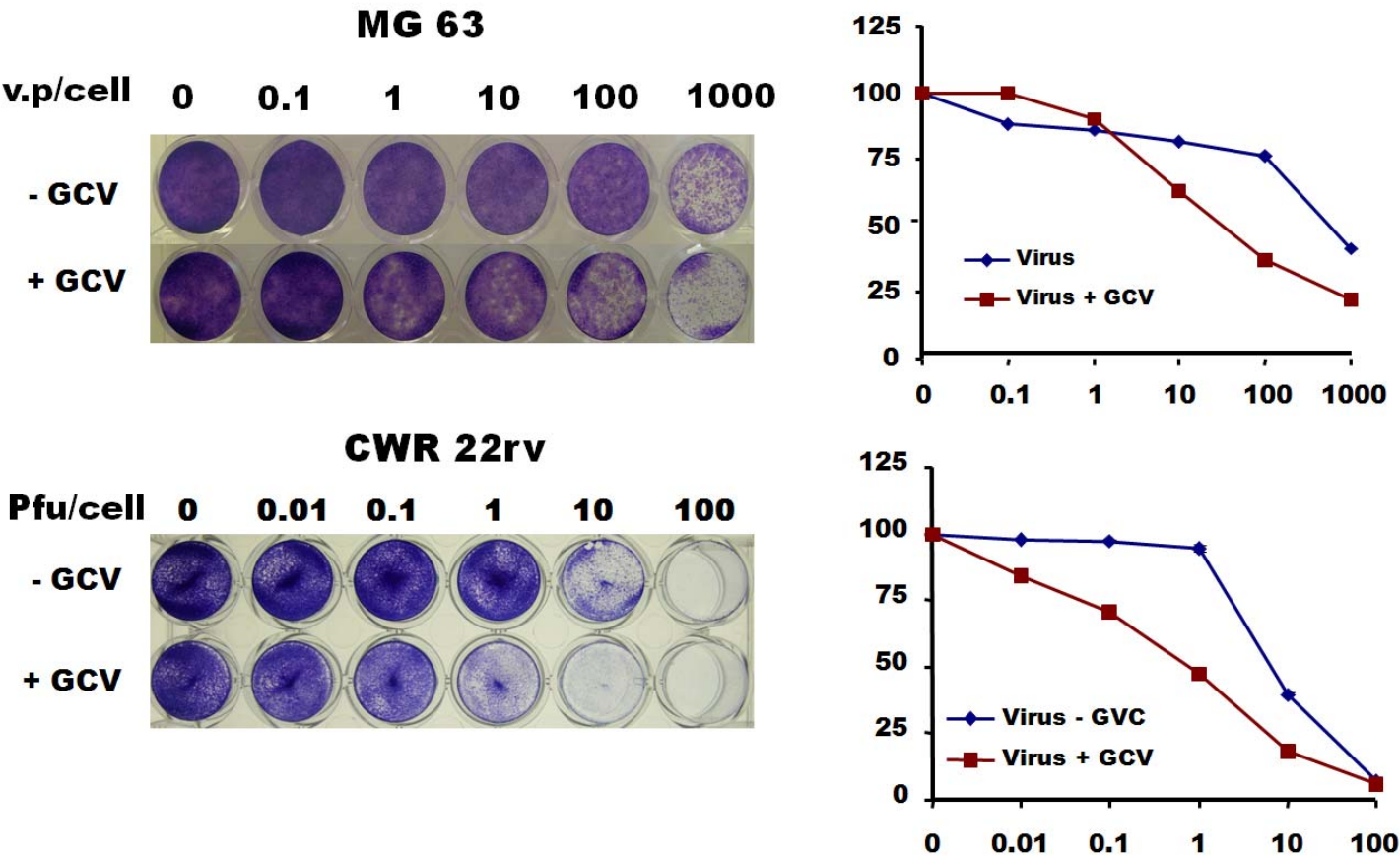


Figure 28. Dose-dependent *in vitro* killing assay. 1×10^5 CWR22rv and 1×10^4 MG cells were seeded in 24-well plates, infected by serial dilutions of AdOCTK from 0.01 to 100 pfu/cell, and then treated with or without GCV (10 μ g/mL). Seven days after infection, cells were stained with crystal violet. Then 1% SDS was added to lyse the cells and for OD₅₉₀ reading. Cell survival rate curves were drawn to evaluate the killing activity of AdOCTK. The growth of AdIU1-infected cells was significantly inhibited by the addition of Ganciclovir (GCV) (10 μ g/mL), especially in CWR22rv.

A. References Cited:

1. Kubo H, Gardner TA, Wada Y, *et al.* Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* 2003;14: 227-41.
2. Hinata N, Shirakawa T, Terao S, *et al.* Progress report on phase I/II clinical trial of Ad-OC-TK plus VAL therapy for metastatic or locally recurrent prostate cancer: Initial experience at Kobe University. *Int J Urol* 2006;13: 834-7.
3. Gambhir SS, Barrio JR, Wu L, *et al.* Imaging of adenoviral-directed herpes simplex virus type 1 thymidine kinase reporter gene expression in mice with radiolabeled ganciclovir. *J Nucl Med* 1998;39: 2003-11.
4. Li X, Zhang YP, Kim HS, *et al.* Gene Therapy for Prostate Cancer by Controlling Adenovirus E1a and E4 Gene Expression with PSES Enhancer. *Cancer Res* 2005;65: 1941-51.
5. Lee SJ, Kim HS, Yu R, *et al.* Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol Ther* 2002;6: 415-21.
6. Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 1997;57: 2559-63.
7. Doronin K, Toth K, Kuppuswamy M, Ward P, Tollefson AE, Wold WS. Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *Journal of virology* 2000;74: 6147-55.
8. Nesterov A, Lu X, Johnson M, Miller GJ, Ivashchenko Y, Kraft AS. Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. *J Biol Chem* 2001;276: 10767-74.
9. Voelkel-Johnson C, King DL, Norris JS. Resistance of prostate cancer cells to soluble TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) can be overcome by doxorubicin or adenoviral delivery of full-length TRAIL. *Cancer Gene Ther* 2002;9: 164-72.
10. Rezacova M, Vavrova J, Vokurkova D, Tichy A, Knizek J, Psutka J. The importance of abrogation of G2-phase arrest in combined effect of TRAIL and ionizing radiation. *Acta biochimica Polonica* 2005;52: 889-95.
11. Pan LC, Price PA. The effect of transcriptional inhibitors on the bone gamma-carboxyglutamic acid protein response to 1,25-dihydroxyvitamin D3 in osteosarcoma cells. *J Biol Chem* 1984;259: 5844-7.
12. Jung C, Ou YC, Yeung F, Frierson HF, Jr., Kao C. Osteocalcin is incompletely spliced in non-osseous tissues. *Gene* 2001;271: 143-50.
13. Wu TT, Sikes RA, Cui Q, *et al.* Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int J Cancer* 1998;77: 887-94.
14. Koeneman KS, Yeung F, Chung LW. Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* 1999;39: 246-61.
15. Bortell R, Owen TA, Bidwell JP, *et al.* Vitamin D-responsive protein-DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression. *Proc Natl Acad Sci U S A* 1992;89: 6119-23.
16. Lian JB, Stein GS, Stein JL, van Wijnen AJ. Regulated expression of the bone-specific osteocalcin gene by vitamins and hormones. *Vitam Horm* 1999;55: 443-509.
17. Banerjee C, Stein JL, Van Wijnen AJ, Frenkel B, Lian JB, Stein GS. Transforming growth factor-beta 1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site. *Endocrinology* 1996;137: 1991-2000.
18. Banerjee C, Hiebert SW, Stein JL, Lian JB, Stein GS. An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. *Proc Natl Acad Sci U S A* 1996;93: 4968-73.
19. Ko ASC, Gotoh G, Kao C, Chung LWK. Tissue targeted toxic gene therapy for an androgen-independent and metastatic human prostate cancer model. *J Urol* 1996;155: 623A.
20. Matsubara S, Wada Y, Gardner TA, *et al.* A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res* 2001;61: 6012-9.
21. Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res* 1999;59: 1498-504.
22. Hsieh CL, Yang L, Miao L, *et al.* A Novel Targeting Modality to Enhance Adenoviral Replication by Vitamin D(3) in Androgen-independent Human Prostate Cancer Cells and Tumors. *Cancer Res* 2002;62: 3084-92.
23. Konety BR, Johnson CS, Trump DL, Getzenberg RH. Vitamin D in the prevention and treatment of prostate cancer. *Semin Urol Oncol* 1999;17: 77-84.

Applicant Name (Last, first, middle): Gardner, Thomas A.

24. Getzenberg RH, Light BW, Lapco PE, *et al.* Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. *Urology* 1997;50: 999-1006.
25. Zhao XY, Feldman D. The role of vitamin D in prostate cancer. *Steroids* 2001;66: 293-300.
26. Koeneman KS, Kao C, Ko SC, *et al.* Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J Urol* 2000;18: 102-10.

Acronyms and Symbol Definitions:

PRRA, prostate-restricted replicate adenovirus; PSES, prostate-specific enhancing sequence; TRAIL, tumor necrosis factor-related apoptosis-induced ligand; PSME, prostate-specific membrane antigen enhancer; PSA, prostate-specific antigen; AREc3, androgen- responsive element; CMV, cytomegalovirus; Luc, luciferase; PSRCA, prostate-specific replication-competent adenovirus; HER, human embryonic retinoblast; EGFP, enhanced green fluorescent protein; GCV, ganciclovir; AP-3, activator protein 3; IFU, infectious unit; AI, androgen-independent; OC, Osteocalcin; LD, lethal dose; AR, androgen receptor; TUNEL, Terminal dUTP Nick End Labeling; MOI, multiplicity of infection; Gy, gray; PI, Propidium Iodide; BGP, bone gamma-carboxyglutamic acid protein; VDRE, vitamin D-responsive element; AML-1, Acute Myeloid Leukemia 1; ACV, acyclovir; CT, computerized tomography;